

Science needs women (cont.)

Campaigns on behalf of women researchers are achieving a higher political profile. Recent European initiatives are welcome, but lobbying leadership is needed for them to be followed through amidst political change.

In recent years the issue of the small number of women working in science has shifted from being seen as one of social justice to one of economic sense — that in societies increasingly demanding a skilled technical workforce we need to stop squandering half of our scientific potential. So the drawing together by the European Commission last week (see page 202) of a so-called ‘network of networks’ of women-in-science groups to form an assemblage large enough, and with enough tentacles, to apply pressure in many of the right places around Europe was a timely idea.

In some places, the women-in-science lobby is already showing the effectiveness of carefully coordinated campaigns; witness, for example, its impact during the recent World Conference on Science, in Budapest. In the United States, the Association for Women in Science (AWIS) has achieved the creation of a Congress-mandated Commission on Women and Minorities in Science and Technology; AWIS’s eight-year campaign has produced a body that will seek to collect examples of effective strategies for recruiting, retaining and advancing women in science.

Last week’s Declaration of Networks Active in Europe sets out ambitious recommendations, its pan-European agenda having its sights firmly set on the political process, arguing for the need for strategic action, lobbying and advocacy. Nevertheless, a notable absence at the commission’s meeting was any emergence of a Europe-based leadership from among the networks. Catherine Jay Didion, the executive director of AWIS, keynote speaker at the meeting and one of those behind the US commission, exemplifies the type of fig-

ure that is urgently required at a European level. Such people are needed to win friends and backers within the science community and to lobby at a political level. A strong figurehead can also gain broader support from the public. The emergence of strong leaders within the women-in-science lobby in the United States and United Kingdom has led to enhanced political clout in those countries.

It is unclear how the issue of women in science will fare in the medium term inside the European Commission. The topic is currently one of considerable activity, following its jump-start last year by the then research commissioner, Edith Cresson. But little is known of the priorities of the proposed new commissioner, physicist Philippe Busquin (see page 203). Given the current momentum behind efforts by the commission on the issue of women in science, this would be an appropriate topic to be pursued by the European Parliament at its hearings on his candidacy later this year.

The recognition of the network of networks is an important step forward for both European science and women scientists. Science needs the best scientists, and that can only be attained by realizing the full potential of women. But although the commission’s first priority might be to use the network to raise the numbers of women in expert advisory panels in the fifth Framework programme of research, it knows that only a small fraction of research spending comes through this route. If the commission is intent on encouraging women in science, it should also seek to stimulate new standards in the national programmes of EU member states. □

Fighting the China syndrome

The next head of the US nuclear weapons laboratories must repair the damage done by the recent spy scandal.

The entire Chinese spy débâcle which has engulfed the United States this year rests largely on a piece of paper detailing aspects of a US nuclear warhead design purportedly stolen by China, which was delivered one day to an American embassy by a “walk-in” source who later turned out to be working for Chinese intelligence.

No one knows why China fed this highly sensitive material back to the United States in this way. Officials at the Los Alamos nuclear weapons laboratory have mischievously suggested that the real intent was to wreak havoc in the United States. They point out, only half in jest, that just such a tactic — the delivery of accurate intelligence to an enemy, followed by the revelation that the source of the intelligence was in fact a double agent — was identified by Chinese military historians in AD 600 as a nifty means of sowing confusion in the enemy camp.

Whatever China was really up to, each twist and turn of the spying scandal — every piece of nonsense spoken about lax security at the laboratories and every clueless intervention from Washington politicians — has added a little credence to the still-implausible theory that the United States is falling victim to a fiendishly clever Chinese plot. In truth, it is difficult to imagine that any enemy action could have deliberately paralysed the US nuclear weapons complex as effectively as this domestically driven scandal has done.

The three weapons laboratories have been in turmoil for most of

the year. Experienced laboratory staff, who have lived and breathed national security for much of their working lives, have been instructed to ‘stand down’ for days at a time, like errant schoolchildren, to listen to politicians’ speeches or read security memoranda. The laboratories’ extensive international connections have been thrown into chaos. Polygraph machines — a kind of embodiment of anti-science, feared by conscientious employees and scoffed at by professional spies such as Aldrich Ames, who passed dozens of polygraph tests — are arriving to keep tabs on some 5,000 laboratory employees.

A Senate proposal in response to the scandal, agreed to last week by Bill Richardson, the energy secretary, would place the weapons laboratories under a semi-autonomous agency within the energy department. But Vic Reis, who formerly ran the weapons laboratories as assistant energy secretary for defence programmes, already enjoyed considerable autonomy within the department. Richardson’s decision to fire Reis — a widely respected research administrator with a strong technical background and good political connections — indicates that scapegoating has gained the upper hand over common sense. The energy secretary says that, if a new agency is established, its chief must have a “national security background”. He or she had better also have the leadership skills and technical credentials to win back the confidence of the laboratories’ tormented employees. □



Legal bid could extend US animal welfare law to cover lab rodents

[WASHINGTON] Lawyers for the US Department of Agriculture (USDA) have been given until tomorrow (16 July) to respond to a lawsuit filed in March by an animal rights group, the Alternatives Research and Development Foundation (ARDF).

The lawsuit, filed in US District Court in Washington, is the strongest challenge yet to the government's contention that some 23 million rats, mice and birds being used in research should not receive protection under the 1966 Animal Welfare Act.

If the USDA loses the lawsuit, the result would be annual, unannounced inspections at laboratories not now under the law's auspices, ranging from small liberal arts colleges whose animal use is confined to mice in psychology departments to labs at biotechnology start-up companies. Universities would be faced with a major increase in paperwork and other compliance requirements.

The lawsuit aims to require the department to extend the protection offered by the act. This explicitly covers dogs, cats, non-human primates, guinea pigs, hamsters and rabbits used in research. But it also applies to any "such other warmblooded animal as the Secretary [of Agriculture] may determine" is being used in research and other activities.

Arguing that this clause gives the USDA discretion on whether to include other animals, the department excluded birds, and rats and mice, whose numbers are exploding in research and which make up some 95 per



In safe hands? Universities and companies in the United States could face \$164 million in extra costs if campaigners succeed in extending to laboratory birds and rodents the legal protection already given to larger animals.

cent of US research animals. But animal rights activists contend that the law is meant to apply to all warm-blooded animals.

The government has been sued before to include rats, mice and birds under the act. In 1992, a federal judge agreed with the Humane Society of the United States and the Animal Legal Defense Fund that the animals should be covered. The judge called the USDA's argument "strained and unlikely". But two years later, an appeals court reversed the decision, saying the plaintiffs had not demonstrated that they had the legal standing to sue.

This time, says John McArdle, the ARDF director, the plaintiffs are optimistic that they

have demonstrated legal standing, which requires showing direct harm to a plaintiff. McArdle says the ARDF, which is based in Eden Prairie, Minnesota, is harmed by the way the USDA has interpreted the law because the group's purpose is to develop and promote the use of non-animal research methods.

The issue is generating considerable heat. During a public comment period that closed in May, the USDA received almost 40,000 comments on a petition submitted last year by the ARDF and others asking it to add rats, mice and birds to the act's protection.

If the lawsuit prevails, paperwork and inspections would increase significantly at most major research universities, which are already covered by the law because of their use of other animals. At the University of Michigan, for example, the change would mean inspections of 51,000 rats and mice, in addition to the 6,000 animals currently examined in an annual five-day visit by the USDA.

Equally important to animal activists, however, is that researchers who use rats and mice would have to demonstrate that they have considered alternative ways of conducting their research. The law requires investigators to demonstrate that they have looked for alternatives, both to painful procedures and to the use of animals altogether. They also have to show that the work does not unnecessarily duplicate previous work. "A lab animal does not have to be the method of choice," says McArdle. "We want to require [researchers] under penalty of law" to consider alternatives.

Research advocacy groups, led by the National Association for Biomedical Research (NABR), argue that extending the law's coverage would cripple the branch of the USDA that is responsible for enforcing

UK spotlight on alternatives to using animals

[LONDON] The British government is to carry out an audit of government-funded research into alternatives to the use of animals in research, before deciding whether to increase investment in this field.

George Howarth, the minister responsible for regulating the use of animals in research, made this pledge at a Home Office meeting last week with both supporters and opponents of the use of animals in research. The audit is expected to be completed by the end of the summer.

Howarth also promised to investigate ways of sharing data from research involving

the use of animals – partly to avoid repeating the experiments. But he acknowledged that commercial organizations may be reluctant to do this.

The meeting, with representatives of more than 50 organizations, was the belated fulfilment of the Labour party's pre-election commitment to look into the use of animals in research. But Howarth remained non-committal on another pledge: to set up a Royal Commission on the issue (see *Nature* 388, 311; 1997).

The meeting brought together representatives of government, scientists, patients' organizations,

industry and animal rights groups. Some questioned the value of convening a large group involving many who are unlikely to change their views.

"Informal contacts can be more fruitful," commented one scientist. The problem, according to another, is that animal rights groups "do not accept the validity of any experiment that involves animals. You can't have dialogue in this situation."

The meeting was organized to explore common ground rather than to reach a consensus, says Howarth. Most agreed on the need to fund more alternatives. **Ehsan Masood**

the law. With a \$9.2 million budget and 65 inspectors — down from 88 six years ago — already covering 10,400 sites, the enforcers are stretched past breaking point, they say.

“Fundamentally we don’t object to the addition of these species, but we don’t want the whole programme compromised,” says Barbara Rich, NABR’s executive vice-president. “I don’t see how it wouldn’t be.”

But critics argue that the USDA’s finances are not the issue. “Justice demands that we treat equal situations equally unless there is a morally significant difference between them,” says Barbara Orlans, a senior research fellow at the Kennedy Institute for Ethics at Georgetown University in Washington, and one of the petitioners. McArdle says the USDA is “using a lack of money as a defence for not doing what they are legally required to do”.

Based on a survey of its members, the NABR concluded that an extra \$84 million in administrative costs would be incurred at institutions already covered by the act. It estimates that, for companies and institutions not now covered, registration and compliance would cost at least \$80 million. The USDA estimated in 1990 that the number of research institutions it regulates would almost double if rats, mice and birds were covered.

The department declines to comment because the issue is under litigation. But the USDA has previously argued that such a change “would have serious consequences for the protection of other species” covered by the law because it would dilute enforcement efforts. It noted that Congress has never moved to include the rodents under the act, although it has amended it several times. “The vast majority of rats, mice and birds used in biomedical research are already afforded certain protections,” it added.

This refers to the fact that researchers who receive Public Health Service funding agree to follow a policy on the humane use and care of laboratory animals that includes all vertebrates. But this policy does not require inspections, nor does it have the force of law.

Animal care administrators say that the requirements of excellent science, and the health service code, mean that rats, mice and birds are already treated as well as if they received protection under the act. “As far as the care of the animals [goes] it wouldn’t make one whit of difference,” says Daniel Ringler, director of the unit for laboratory animal medicine at the University of Michigan.

But a survey last month in *Lab Animal* turned up surprising results from members of Institutional Animal Care and Use Committees, the panels of veterinarians, researchers and other faculty members responsible for ensuring that their institutions comply with animal welfare rules. Of 491 committee members surveyed, 73 per cent said that rats and mice should be covered by the act. The same proportion was true for the 287 animal researchers in this group. **Meredith Wadman**

Five young life scientists win \$1m no-strings grants

[WASHINGTON] Five young US biologists have been chosen as the first recipients of \$1 million each by the W. M. Keck Foundation. They can use this money to pursue exciting ideas as they see fit, free of the restrictions that usually accompany research grants.

Bruce Clurman of the Fred Hutchinson Research Center in Seattle, Judith Frydman of Stanford University, Partho Ghosh of the University of California at San Diego, Phyllis Hanson of Washington University, St Louis, and Mark Gerstein of Yale University were informed last week that they are to receive the first five awards in Keck’s programme. There were 24 nominations by various biomedical research centres.

Frydman, a 35-year-old who will use her award to investigate protein folding, says that it will offer far more flexibility than a grant from the US National Institutes of Health, for which an outline of the proposed work has to be planned in advance. “This will allow me to start asking new questions, where I’m not necessarily sure what is going to be the right approach,” she says.

Ghosh, a 36-year-old structural biologist, says his award will support “a lot of projects

that a young professor might otherwise have difficulty taking on”. He added that his laboratory will investigate proteins that are developed by pathogens to breach the membranes protecting host cells.

The foundation launched the programme last year, after deciding that young biomedical researchers at the peak of their creativity were being hemmed in by the strings attached to conventional grants. It intends to select five more young professors for each of the next four years, spending \$25 million in all. The awards are open to US citizens who have held faculty positions for no more than three years and who are investigating “fundamental mechanisms of human disease”.

According to William Butler, chancellor of Baylor College of Medicine in Houston, Texas, and chair of the advisory panel for the programme, they are also intended to address what he terms the “incredible funding pressures” at US medical schools caused by sharp reductions in hospital bed income.

The W. M. Keck Foundation was set up 45 years ago by the founder of the Superior Oil Company, and is now one of the largest philanthropic organizations in the United States, with \$1.5 billion of assets. **Colin Macilwain**

UK biotech industry aims to clean up its act

[LONDON] The leaders of Britain’s biotechnology industry have decided to get tough with companies that bring the industry into disrepute, issuing a draft code of practice for companies in the medical and life sciences sector.

The code is aimed at managing the flow of scientific information to shareholders in a way that does not adversely affect a company’s share price, nor falsely raise expectations from patients’ groups.

The nine-point code, released for public comment last week by the BioIndustry Association (BIA), is also aimed at helping bioscience companies to avoid the fate of the deeply troubled, but one-time flagship company, British Biotech.

The company was censured last month by the London Stock Exchange and the US Securities and Exchange Commission after an investigation concluded that it had issued misleading assessments on the status of its drugs trials (see *Nature* 392, 852; 1998).

While the code is not mandatory, all of the BIA’s member companies listed on the Stock Exchange will be asked to provide information on the state of compliance in their annual reports. Companies will have to provide reasons for non-compliance. The

identity of those who refuse to comply will be made public. Some could even be expelled.

The code has been enthusiastically received by many companies in Britain’s 460-strong bioscience sector, as well as by the Stock Exchange and the Association of British Insurers, which represents the single largest shareholding community.

“The financial community expects company information to be full, frank and open. That’s what the code hopes to achieve,” says BIA chief executive John Sime.

“Investors tell us they will take a dim view of companies who do not comply,” says Robert Mansfield, BIA’s chairman. “In the life sciences, there has to be a partnership between management and shareholders. It is a long-term relationship based on trust.”

The code’s provisions include: ensuring that the boards of companies have access to independent scientific advice, and expertise in handling scientific information; making sure that information to investors is as transparent and accurate as possible, and avoiding any temptation to oversell the implications of a result. The code also asks company scientists to exercise caution when discussing potentially price-sensitive information with peers. **Ehsan Masood**

France losing genome race, says report ...

[PARIS] The gap between France and its competitors in genome research will widen even further unless research is stepped up, particularly in bioinformatics, according to a report published last week by the French Academy of Sciences.

In particular, it calls for the strengthening of the Génopôle biotechnology park at Evry near Paris, and wants a national network of similar initiatives to be built "as fast as possible" in cities such as Lille, Strasbourg, Marseille and Montpellier.

The report points out that, although France had a head start on genome research by publishing the first physical and genetic maps of the human genome in 1992 (see *Nature* 359, 794–801; 1992), it has failed to keep pace with American and British efforts.

"France cannot catch up in the realm of sequencing, but at the post-genome level there are steps that can be taken," says François Gros, the permanent secretary of the academy and a former director of the Institut Pasteur, who coordinated the 230-page report.

This isn't the first report in recent years that has questioned France's approach to biotechnology research. A report commissioned last year by Claude Allègre, the minister for national education, research and technology, and Dominique Strauss-Kahn, the industry and finance minister, touched on similar issues (see *Nature* 392, 214; 1998). The findings lamented the fact that archaic



Gros: 'steps can be taken' to help France catch up.

state industrial policies and a dearth of start-up companies were holding France back in the international arenas of biotechnology and computing.

The new report, drawn up by 22 scientists, was also commissioned by Allègre. It calls for a rapid overhaul of genome research in France if the country is to continue to make significant advances. And it describes the

French genomics industry as insufficient, calling for more support for start-up companies along the lines of Genset and Transgène.

Panel member Jean-Michel Claverie, director of the Information Génétique et Structurale unit at the Centre National de la Recherche Scientifique, who was responsible for the report's section on bioinformatics, argues that this is one of France's greatest weaknesses in genomic research. While this science has been developing rapidly in the United States and the United Kingdom, France has been slow to update its more theoretical approach to computer

sciences, he says.

The limiting factor to the development of bioinformatics is a lack of trained professionals, he adds. Heavy recruitment by large companies such as Glaxo and Merck have "just created a vacuum. There aren't enough professors to start programmes and teach courses."

Other underdeveloped areas, the report concludes, are academic programmes geared towards genomics and collaboration between laboratories and clinicians. Research into plant and agricultural genomics and structural biology also needs strengthening.

With these tools at France's disposal, the report indicates, the country could become involved in some of the applications of post-genome research, in medicine, pharmacology and agriculture. If it does not, the report warns, the country may find itself technologically incapable of taking the next step in the genomic revolution.

Citing the example of bioinformatics, the report states: "Not having enough bioinformaticians in France or in Europe means that the sequences we produce at great cost will be analysed, interpreted and exploited by others, principally the United States".

The document, entitled *Development and Applications of Genomics, After the Genome*, is the first of a series of biannual reports on the state of science and technology to be commissioned from the academy.

Heather McCabe

... as government announces creation of genome research consortium

[PARIS] The call for an overhaul of genome research in France (see above) coincided with the announcement last week by the Ministry of Science, Education and Technology of the creation of a consortium for genome research.

The consortium will link private companies, public research units and university groups, and is set to receive FF1.5 billion (US\$235 million) of government funding over the next three to five years.

The money will go towards research at France's genome laboratories, and towards building a national network of 'génopôles' – clusters of genome-related laboratories and companies – similar to that being built at Evry, near Paris. How the companies will contribute to the consortium – either financially or by releasing some of their results for use by other members of the consortium

– is still under negotiation.

It is widely accepted that French genome research could benefit from increased government support. But early proposals for the use of the extra money kindled controversy over reports that up to half would be earmarked to support the company Genset, France's main hope in industrial genome research, which has seen the value of its stock-market shares halved in the past 12 months (see *Nature* 399, 185; 1999).

No announcement has been made on how the money is to be allocated. But geneticists close to the issue say that, since the allegations of a possible government subsidy to Genset, the ministry has broadened the consortium to include more companies and research centres.

Plans now call for the involvement of Synthélabo, Sanofi

and Rhône-Poulenc, as well as France's Institute of Genetics and Molecular Biology near Strasbourg, Génopôle and the Evry-based National Sequencing Centre (Genoscope) and National Genotyping Centre.

Regardless of whether Genset will be a major recipient of the funding, some scientists criticize the decision, saying that taxpayers' money should fund public research units whose budgets are inadequate, rather than companies.

"The government should give money to public research units such as CNRS (Centre National de la Recherche Scientifique) and INSERM (the national biomedical research agency), who in turn could invest in contracts with the private sector," says Jean-Michel Claverie, director of the Information Génétique et Structurale unit at CNRS.

Claverie says the consortium raises questions of who will publish research data and who will have access to it. "I think a merger between public and private interests is confusing a lot of issues, and may be considered unfair competition by companies outside the consortium and in the United States and United Kingdom," he says.

Others note that the consortium is an example of France's tradition of using public funds to support private industry. In France, "if the state does nothing, no one will do it," says Bernard Dujon, professor of molecular genetics at the Institut Pasteur in Paris.

Claverie points out that, although the report from the Academy of Sciences calls for an overhaul of genome research, such a consortium was not what the authors had in mind. **H.M.**

Bumpy ride for 'core e-journals' project

[SAN DIEGO] A bold experiment in electronic use of scientific journals by the California State University (CSU) system has met mixed responses since its launch on 1 July.

CSU entered an 18-month contract with a secondary publisher (a subscription service that sells packages of journals to libraries, under licence to the publishers) for a 'core' set of electronic subscriptions, in a bid to improve access and cut costs. This was to include the journals most requested at two-thirds of campus libraries.

But the university, which offers mainly undergraduate and masters degrees, is getting far fewer journals than it wanted and the future of the project now looks uncertain.

The project, set up by a committee consisting solely of California State librarians, is widely seen as a radical move with little faculty input. A university spokesman said "The librarians know what the faculty are looking for", and committee member John C. Calhoun, librarian at the Dominguez Hills campus, claimed it was not in the nature of librarians to engage in "radical moves to imperil scholarship". But researchers and students

are expected to clash with librarians over reduced availability of research material.

When proposals were requested last winter, university officials sought some 1,300 journals for the 21 libraries at the statewide system, which has 45,000 faculty members and about 350,000 students. Alabama-based EBSCO Information Services won the \$500,000 contract. But the libraries will only receive about 500 'core journals', as EBSCO could not persuade others — including many of the major names such as *Nature*, *Science*, *Cell*, *Biochemistry* and *Geology* — to join the agreement. Campus libraries will have to subscribe to these individually.

David Kerin, a general manager of EBSCO, said this had happened because the university "didn't want to spend more money, and certain publishers didn't want to play ball by California's criteria".

CSU officials say the contract still represents a strong political statement, wresting control over the maintenance of academic journal collections from publishers and secondary publishers. These have forced many libraries to buy packages, including some

journals they don't need and multiple copies of others when they only required one.

Evan A. Reader, director of CSU's electronic information services, said the contract was the first major step towards moving libraries to electronic publishing. The deal also secured some concessions, including perpetual use of articles the university has paid for (even if the subscription is later cancelled), unlimited interlibrary loans and occasional use by authorized members of the public at university libraries.

This contract is among a number of moves sweeping the research-publishing world as use of the Internet increases. The National Institutes of Health (NIH) is proposing its own electronic publishing system to counter what it considers high costs to universities it funds. Stanford University and the *British Medical Journal* recently announced plans to publish electronic journals of non-peer-reviewed research (see box). And the University of California's Digital Library has just signed a long-term contract with Elsevier Science to provide most of the Dutch firm's 1,100 journals to its campuses through an electronic system, ScienceDirect.

While CSU's experiment is being monitored by institutions as far away as Japan, there are considerable obstacles to its success — in particular, the difficulties libraries face in securing the journals they want from publishers reluctant to change the rules. Elsevier has already declined to provide any through the California State contract. Elsevier spokesman John Tagler said the university system wanted only 27 Elsevier journals for its core group, whereas the university libraries had been subscribing to nearly 400.

In future, each CSU library must buy any extra journals from its own budget. This sets the stage for an inevitable fight over library expenditures. The core-journal contract was funded from a special state library allotment; when it ends university officials will have to determine new funding mechanisms. Meanwhile Calhoun expects libraries to begin the "time-consuming and anxious process" of deciding what print journals to cancel next year when renewals become due. He says there will be "advise and consent" with faculty during this transition.

At present, campuses remain wary. San Diego State University (SDSU), which has the system's largest and oldest library, won't consider dropping its 5,000 print subscriptions until the core-journal programme establishes a credible track record over a considerable period of time. "We are taking a wait-and-see approach," says the library's interim dean Karen Kinney, adding that gaps in its collection would be costly and problematic to fill if it relied too much on the developing programme.

Rex Dalton

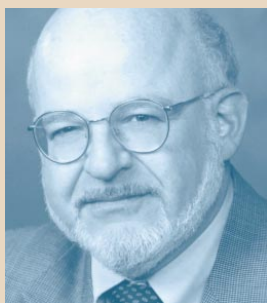
Critics query financing of proposed 'E-Biomed'

[WASHINGTON] The new president of the largest lobby group representing US biomedical researchers last week joined critics of the planned website 'E-Biomed'.

Intended as a free, unifying site for biomedical literature, 'E-Biomed' has been proposed by Harold Varmus, director of the National Institutes of Health (NIH). But David Kaufman, who took office this month as the president of the Federation of American Societies for Experimental Biology (FASEB), says its financing has not been thought through.

He adds that 'E-Biomed' will not be 'free', since there will be irreducible production costs including review, editing and layout, even if users don't pay for access.

"They haven't listed a variety of approaches for paying for this," Kaufman said. "If it were more thoroughly fleshed out, the discussion about it could be engaged with facts, instead of the worst-case scenarios on everybody's mind."



Kaufman: site will not be free.

Varmus intends 'E-Biomed' to allow fast access to the entire biomedical literature by anyone with a computer and an Internet connection (see *Nature* **398**, 735; 1999 and **399**, 8-9 & 720; 1999). He has been dogged by the question of how it would be financed, but said recently that "this very important issue" would need to be thoroughly addressed by a proposed international governing body.

Many decisions on the matter, he wrote on NIH's website, were likely to be left to individual journals and publishers. But one of Varmus's suggestions —

authors' fee of \$200-\$1,000 per article — wouldn't come close to financing the site, says Kaufman, a professor of pathology and laboratory medicine at the University of North Carolina at Chapel Hill.

Kaufman adds that officials from FASEB, whose 19 member societies publish some 35 journals, have "talked about thousands of thousands as a likely cost" per article to authors if their fees were to entirely underwrite 'E-Biomed'.

Michele Hogan, the executive director of the American Association of Immunologists, which publishes the *Journal of Immunology*, estimates that the journal's \$60 page charge would increase about five-fold for authors if they became the means by which the system was financed.

Kaufman suggested that 'E-Biomed' might be funded completely by NIH, or that time on the system could be metered, with users paying for access according to the time spent. **Meredith Wadman**

US seeks a big increase in wind power

[BOSTON] The Clinton administration has announced a major initiative to stimulate the use of wind as an energy source. In particular, it has set a goal of increasing the proportion of electricity produced by wind turbines from 0.1 per cent of the national total to 5 per cent by 2010.

Addressing the annual meeting of the wind-energy industry in Burlington, Vermont, last month, energy secretary Bill Richardson unveiled the Wind Powering America programme, which calls for dramatically increased reliance on wind energy.

The programme is to be funded through budget requests for the Department of Energy (DoE) and other federal agencies, as well as private-sector investments.

"Wind energy has been the fastest growing source of energy in the world during the past decade and now represents a major economic opportunity for the United States," said Richardson. He pointed out that the power harnessed from wind world-wide exceeds 10,000 megawatts.

He added that the programme "will help combat global climate change by reducing carbon emissions". The greater use of wind energy, he said, could lead "the charge in the transition to renewable energy".

The federal government has promised to increase its own use of wind power to 5 per cent by 2010 by installing wind turbines purchasing wind-generated electricity.

Richardson said that research and development into wind power should be expanded to halve the cost of electricity from wind generators. He also announced \$1.2 million in energy department grants to ten states to support wind-energy projects.



Blown along: Kansas residents inspect the state's first two wind generators, dedicated last month.

The proposals were enthusiastically greeted in Burlington. "This is the most visible and aggressive commitment to wind energy I've seen in my 20-plus years in the industry," says Randall Swisher, executive director of the American Wind Energy Association, which sponsored the four-day conference, Windpower '99.

Tom Gray, the group's deputy director, says the programme "recognizes that wind is the biggest success story in the renewable-energy field, and the closest to being commercially competitive".

Wind turbines produce electricity at about 5 cents per kilowatt hour, roughly an eightfold drop since 1980, according to Richardson. Costs have declined as turbines have become larger, more efficient and more reliable. "Early generations of turbines had a variety of problems that we've gradually

been able to eliminate," says Dan Reicher, assistant secretary for energy efficiency and renewable energy at the DoE.

But wind still needs some help to narrow the gap with the cheapest fossil-fuel sources, says Reicher. The DoE is working with manufacturers to cost cuts by designing lighter turbines with improved aerodynamics and enhanced power-conversion systems.

The department also endorses a package of financial incentives to promote wind use. A production tax credit expired on 30 June, but the Clinton administration hopes to extend this and has backed a bill requiring electricity suppliers to use renewable sources for 7.5 per cent of the power generated by 2010.

The DoE plans to sponsor several studies, including an assessment of power transmission capacity, an analysis of wind resources throughout the country, and siting reviews to ensure that birds would not be harmed by turbines. Training programmes will be set up for wind-turbine engineers and technicians.

The initiative also calls for a tripling in the number of states with significant wind-power capacity (exceeding 20 megawatts) by 2010. DoE officials do not expect major objections to the technology on noise or aesthetic grounds, as most installations should go up in remote agricultural land.

Wind turbines could provide major economic benefits to farmers, says Reicher, and could serve as a new 'cash crop'. In time, the United States can go well beyond capturing 5 per cent of its electricity from wind, he adds.

But the administration must first secure the funding to advance wind technology. Wind might then become the major power source its proponents envisage. **Steve Nadis**

Mexican project shows that emissions reductions could be traded

[WASHINGTON] A pilot project involving Mexico and Norway has shown that reductions in greenhouse gas emissions from developing countries can be satisfactorily measured and certified for 'trading' purposes.

Under the \$23 million Ilumex project, residents in two Mexican cities, Guadalajara and Monterrey, replaced millions of ordinary light bulbs with energy-efficient fluorescent bulbs that use a quarter of the power and last ten times as long.

Auditors say the move generated energy savings that reduced carbon dioxide emissions from Mexican power stations by more than 170,000 tonnes from 1995 to 1998. The reduction was certified by Det Norske Veritas, an independent foundation based in Oslo, with assistance from the US engineering consultancy ICF.

Sceptics argue that such reductions are

difficult to measure and that international trading in emissions credits will be prone to fraud. But Kristian Holthe of Norway's ministry of foreign affairs, announcing the certification at the World Bank in Washington last week, said the pilot shows that it is possible to measure and certify emissions reductions attributable to particular projects. He said it "bodes well for the implementation of the Kyoto Protocol" to reduce emissions.

The parties to the protocol have yet to reach agreement on mechanisms for trading emissions credits. For example, developed nations may be able to obtain credits towards meeting emissions targets by helping developing nations to reduce their emissions.

The Global Environment Facility — an offshoot of the United Nations and the World Bank — paid \$10 million towards Ilumex, a sum matched by the Mexican

electricity company Comision Federal de Electricidad, while the Norwegian government contributed \$3 million.

Most of the money was used to subsidize the sale of the more expensive fluorescent light bulbs. Auditors checked the sale and installation of the bulbs, their performance and their effect on carbon dioxide emissions.

Bob Watson, environment director at the World Bank, added that arrangements for trade or exchange of emissions credits were unlikely to be agreed until the sixth meeting of the conference of the parties to the United Nations Framework Convention on Climate Change, expected to be held late in 2000.

Supporters of emission controls hope that such a framework will open the way for global compliance with the Kyoto Protocol. But the US Senate remains hostile to the protocol, and is not expected to ratify it in the near future. **Colin Macilwain**

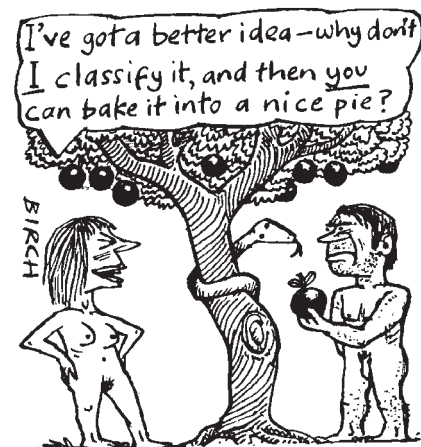
Supernetwork to boost women in science

[BRUSSELS] Representatives of women in science groups across Europe and other bodies committed to improving the gender balance in scientific decision-making agreed last week to work together as a 'network of networks'.

A meeting in Brussels of 70 organizations making up the 'supernetwork' issued a joint statement of common goals and objectives. This described the under-representation of women in science as "a serious obstacle for the development of the sciences and for European society".

It called for all institutions that employ scientists to produce annual statistics on gender monitoring, and suggested increased lobbying and advocacy on the need for more women in science at national and European level.

Those attending the meeting — held under the auspices of the European Commission's research directorate, DGXII — also proposed that the commission should use the network to document best practice in member states of the European Union. Fur-



ther support for their joint activities will be sought from the commission.

Nicole Dewandre, of DGXII's Women and Science sector, says that the joint declaration is intended to establish the identity of the 'network of networks' before it starts "knocking on doors".

One of the goals of the Brussels meeting had been to bring those concerned about

gender issues in science into the commission's fifth Framework programme of research (FP5). "But we also wanted to ask what, as the commission, we could bring to them," says Dewandre.

The commission has undertaken to make "significant efforts" to increase women's participation in the union's research programmes. The overall objective is to achieve for women at least 40 per cent representation, on average, throughout FP5, including Marie Curie scholarships, advisory groups and assessment panels.

One hope for the meeting is that it will lead to an exchange of experience between the 'network of networks' and a separate network of government officials in member states involved in promoting women in scientific research. In the autumn both groups will consider a report on the challenges to women's participation in European research policy and put forward recommendations. This report is being produced by a group of experts set up last year by the commission. "There is a growth of awareness of the need for strategic action," says Dewandre.

The new network has been set up as part of the commission's efforts to establish links with Europe's existing networks of women scientists. The commission is especially keen to see more female applicants for 'expert positions' in advisory groups and assessment panels within FP5. The current figure stands at around 15 per cent.

The representatives of networks told the commission that it should consider paying higher salaries to researchers with families to support. They suggested that the age limit for fellowships should be relaxed to take into account academic years completed, rather than age — a move that would help women who had taken time out to raise a family.

But Achilles Mitsos, director of DGXII, gave little ground when fielding such suggestions in an open session. This was "not the time to change council decisions," he said. "We should exert pressure, but this will come a little later."

Although some were "not optimistic" about Mitsos's reaction, all were positive about the outcome of the meeting and what could be gained by forming the network.

In a keynote speech, Catherine Jay Didion, executive director of the US Association for Women in Science, showed how the lobbying of politicians can be carried out effectively. The association has been largely responsible for the creation of the Congress-mandated Commission on Women and Minorities in Science and Technology.

For some, the meeting itself was a revelation. "It has changed my life," says Sue Black of the British Computing Society. "I didn't know all these women were out there." **Natasha Loder**

NASA to visit Mercury and dig into a comet

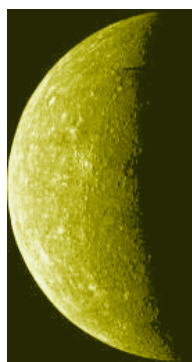
[WASHINGTON] A return to the innermost planet Mercury and a mission to excavate material from a comet's nucleus were selected by the US space agency NASA last week as the next in its Discovery line of planetary missions.

The \$286 million Mercury Surface, Space Environment, Geochemistry and Ranging (Messenger) mission and the \$240 million comet impactor, called Deep Impact, beat three other finalists in the funding competition. Those were a sample return from the Martian moons Phobos and Deimos, a Jupiter orbiter, and a spacecraft to study the atmosphere of Venus.

A return visit to Mercury has been a perennial on the wish list for Solar System missions. Messenger will launch in 2004, 30 years after Mariner 10 made two quick fly-bys, the only reconnaissance of the planet to date. The spacecraft will arrive at Mercury in 2008, carrying seven instruments for imaging, spectrometry, laser altimetry, and measurements of the planet's magnetic field.

During its year in orbit, it will map the surface, including the hemisphere that Mariner missed. Messenger's principal investigator is Sean Solomon of the Carnegie Institution of Washington, and the spacecraft will be built and managed by the Johns Hopkins University Applied Physics Laboratory.

NASA's Jet Propulsion Laboratory will manage Deep Impact. This will create its own fireworks on 4 July 2005, Independence



Messenger will map Mercury's surface.

Day in the United States, when it smashes a 500-kilogram copper projectile into Comet P/Tempel 1 at a velocity of ten kilometres per second.

The impact will gouge out a crater at least 25 metres deep and 120 metres in diameter — deep enough to expose pristine material from inside the comet, hopes principal investigator

Michael A'Hearn of the University of Maryland. Cameras on the main spacecraft will image the evolution of the crater and the resulting plume in infrared and visible wavelengths.

A'Hearn was instrumental in organizing a ground-based campaign to observe the 1994 collision of comet Shoemaker-Levy 9 with Jupiter. He envisages a similar marshalling of professional and amateur telescopes to watch the artificial collision.

Deep Impact features another first for the Discovery programme — the offer of a 'piggyback' ride to other investigators. A'Hearn's team have an extra 10 kilograms of mass and 10 watts of power on the spacecraft, which it will make available for a technology experiment to be selected at a later date.

Tony Reichhardt

Spanish university sued for advertising posts in August

[BARCELONA] A Spanish university is being sued by a scientists' association for having advertised 80 posts for professorial appointments last August, a month when many Spanish citizens — and thus potential external applicants — are on holiday.

The Spanish Association for the Advancement of Science and Technology (AACTE) argues that this practice is being deliberately used by the University of La Laguna (ULL) in the Canary Islands to favour the appointment of local candidates. It thus undermines efforts to improve the quality of research in Spanish universities.

In response to the lawsuit, the university says that the practice makes use of administrative resources that would otherwise be underused during the summer. But the university's vice-chancellor has said that it will examine whether things could be done differently in future.

In its lawsuit, the association points out that the strategy of advertising professorial posts in August has been used by the university for several years. In 1997, for example, ULL announced about 60 professorial positions during this period.

The advertisement for applicants for posts in 1998, the last year listed in the lawsuit, was officially issued by the university on 24 July. But it was not published in the daily *Official State Bulletin*, which publishes all official notices involving Spanish public institutions, until 14 August, reflecting the frequent delay between an institution's decision to advertise and the advertisement's publication. The notice gave applicants 20 working days in which to file their applications with the university.

Economist Ruth Rama, deputy director of the Institute of Economy and Geography in Madrid, and treasurer of the AACTE, argues that advertising university posts during holiday periods is a restrictive practice often intended to favour local candidates.

She says the university may have deliberately chosen the dates of the advertisement to minimize the number of external

candidates, and that such practices "violate the constitutional right of equal opportunities for access to public functions that all citizens have".

Rama points out that most public libraries in Spain are closed in August, preventing potential external candidates from reading the daily bulletin. "A virtually total lack of information of the posts is guaranteed," she says.

In a formal response to the AACTE lawsuit to the Canary Islands Superior Court, ULL's legal representative, Miguel Rodríguez Berriel, argues that there is no rule forbidding the universities from advertising posts in August. The university's administrative activities "cannot stop in the holiday period, as that would mean a waste of educative public services".

The AACTE was set up in 1997 by Spanish researchers concerned that Spain was failing to take the steps needed to bring its science up to the level of most advanced countries. One of its main objectives is to monitor the selection procedures for academic positions, and to check whether appointments are made on the basis of academic merit and equal opportunity.

One of its vice-presidents, Luis Rull, professor of physics at the University of Seville, says it is "very appropriate" that ULL has been sued for advertising professorial posts in the summer holiday periods. He says that such tactics undermine "the goal of looking for the best researchers for our universities, and that ULL officials are clearly harming the Canary Islands Autonomous Community".

Rull suggests that university departments in Spain should be ranked according to their research output "so that Spanish citizens are aware of which are the best ones to study at". If not, he says, there will be little pressure to change, as "it may be irrelevant for society that a university tries to appoint mediocre candidates, or even local candidates who have never done research".

The university's vice-chancellor, José Gómez Soliño, denies that the university is trying to hide the posts by advertising them in August. He points out that the university has used the same practice every year, and argues that it is therefore "a problem of bureaucracy" that the advertisements are always published in August.

Gómez Soliño says ULL is not the only Spanish university to advertise professorial posts in August. But he adds that the university intends to study possible ways of avoiding this in future. "We can change things in order that posts are not advertised in August," he says.

Xavier Bosch



Busquin: solid background in science and politics.

Belgian scientist proposed to head EC research directorate

[BRUSSELS] A former physicist who took postgraduate degrees in philosophy and environmental studies before turning to politics has been nominated as head of the European Commission's research directorate, DGXII.

Philippe Busquin, leader of Belgium's French-speaking Socialists, is one of a team of proposed commissioners announced last week by Romano Prodi, the president-designate of the European Commission.

All candidates will have to attend individual confirmation hearings with the European Parliament in late August. Centre-right MEPs have already voiced criticisms over the left-wing emphasis in Prodi's proposed cabinet.

But Prodi denies any bias. "This is a top-quality team in which jobs have been allocated to match the proven abilities and experience of each commissioner," he says.

Busquin, with a solid background in both science and politics, seems well placed to take responsibility for the commission's research programmes, including in particular the fifth Framework programme that began this year. After graduating in physics from the Free University of Brussels (ULB) in 1962, he took a two-year degree in philosophy, followed by postgraduate studies in environmental issues, also at ULB.

He was an assistant lecturer in physics at ULB between 1962 and 1977, while also teaching at Nivelles Teachers' Training College. From 1978 to 1980 he was chairman of the board of directors of the Institut National des Radioéléments in Fleurus.

Elected to the Belgian parliament in 1978, Busquin has been president of the country's Socialist Party since 1992 and held various ministerial positions before being elected a senator in 1995. He was elected to the European Parliament last month.

Natasha Loder



Hiding in the sun? The University of La Laguna.

Public fears halt bioterrorism tests in New Mexico

[WASHINGTON] The planned release of a common bacterium into the air at Los Alamos National Laboratory to test new biodetector technology was cancelled last week, owing to public concern over safety.

The bacterium, *Bacillus globigii*, is found naturally in soils and is commonly used as a surrogate for dangerous pathogens in germ warfare studies. Despite assurances by Los Alamos officials and the New Mexico Department of Health that its release posed no public health threat, local people and activist groups spoke out against the tests at a meeting on 7 July in nearby White Rock.

"Maintaining the trust of our neighbours is essential," said Don Cobb, associate laboratory director for threat reduction. The biodetector tests, part of an effort to improve US capability to respond to terrorist attacks, will be moved to another site.

Italian satellite missions cleared for take off

[MUNICH] The Italian Space Agency (ASI) has given final approval to a series of small scientific satellite missions selected by the

Italian scientific community. The first launch, planned for 2001 or 2002, will be a gamma-ray astronomy mission called AGILE (Astrorivelatore Gamma ad Immagini Leggero), budgeted at IL53 billion (US\$27.7 million). AGILE will image the gamma-ray sky in the tens of MeV range, combining high sensitivity, angular resolution and breadth of field view.

The next mission is a high-frequency data transmission satellite called DAVID (Data and Visual Distribution), budgeted at IL74 billion. Set in a polar low-altitude orbit, it will cover the Earth's surface and help develop suitable systems of data transmission from remote research stations, for example in Antarctica, or from remotely sited telescopes. It will also study high-frequency signal propagation through rainclouds.

French advise caution on xenotransplants

[PARIS] A French ethics body has said it is too early to proceed with clinical trials of xenotransplantation — the transplanting of animal cells, tissues and organs into human beings. But the National Consultative Ethics Committee for Health and Life Sciences has held back from demanding a moratorium similar to that called for by the Council of Europe this year (see *Nature* 397, 281; 1999).

The committee's report says it would be premature to proceed with clinical trials without knowing more about the potential risks of introducing infectious diseases into human beings. It encourages further research, noting that xenotransplantation represents a "serious hope" for solving the shortage of human organs.

More teeth for watchdog on research risks

[WASHINGTON] The Office for Protection from Research Risks (OPRR), the watchdog body responsible for overseeing human subjects research in the United States, is to be upgraded and moved from the National Institutes of Health (NIH) to the office of the health secretary. The change, suggested by the advisory committee to the director of the NIH last month, was announced last week by Donna Shalala, Secretary of Health and Human Services.

Shalala said that the directorship of OPRR would be upgraded and its resource needs reviewed. Pressure has been mounting to strengthen the OPRR, as evidence accrued that it lacked the authority or the resources to police the hundreds of local institutional review boards that are supposed to protect patients involved in clinical research (see *Nature* 397, 550; 1999).

Hormone patent case set for retrial next year

[SAN DIEGO] A new trial will be held in January on the University of California's lawsuit against Genentech, Inc. for alleged patent infringement on a growth hormone drug, a federal court in San Francisco ruled last week. The first trial ended in deadlock. Eight of the nine jurors decided in favour of the university, but it needed a unanimous verdict to win (see *Nature* 399, 512; 1999).

The stakes in the new trial will be even higher, as the judge has decided it will address Genentech's alleged infringement involving two growth hormone drugs as well as the question of 'wilfulness'. Attorneys say this will raise the potential damages award in the nine-year-old case to \$1.5 billion. The original trial involved one drug, Protropin, but the new trial will also include Nutropin.

Battery failure spells doom for X-ray satellite

[MUNICH] The German X-ray satellite *Abrixas*, whose battery system failed two days after its launch in April, cannot be reactivated. Experts from the German space agency DLR announced the death of the mission after it proved impossible to restore *Abrixas*'s main battery during the satellite's

six-day sunlight period at the end of last month (see *Nature* 399, 93; 1999).

Abrixas was designed as a relatively cheap mission. Supplied with a high-tech X-ray camera, it was to have carried out the first all-sky survey in the X-ray range.

Australia strengthens research ties to Europe

[BRUSSELS] The European Union (EU) and Australia last week agreed to enlarge their scientific collaboration. In signing an amendment to a 1994 agreement, the first signed by the union and an outside country, both sides agreed to widen the field of their scientific and technological cooperation to all thematic programmes of the EU's fifth Framework research programme.

Future collaboration will include research carried out on large-scale facilities in EU countries. In return, European researchers will be given access to Australian research programmes. Edith Cresson, the EU research commissioner, said the collaboration should serve as a model for future agreements.

Ex-NIH boss moves to American Red Cross

[WASHINGTON] Bernadine Healy, 54, the cardiologist who directed the US National

Institutes of Health (NIH) from April 1991 until June 1993, was named last week as president and chief executive officer of the \$2.3 billion American Red Cross. She will succeed Elizabeth Dole, who is now a potential Republican presidential candidate.

Healy will leave her current job as dean of the Ohio State University College of Medicine and Public Health in Columbus to assume her new post on 1 September. She will be the first physician to preside over the charitable organization, the country's largest blood supplier and provider of disaster relief.

Wagner heads German particle physics centre

[MUNICH] Albrecht Wagner, a 58-year-old experimental physicist, is to take over as head of Germany's national research centre for particle physics (DESY) in Hamburg. He succeeds Björn Wiik, who died in a domestic accident in February (see *Nature* 398, 7; 1999).

Wagner has been a director of research at DESY since 1991, and is an expert on the physics of electron-positron linear colliders such as the TESLA facility, a 33-km superconducting linear accelerator proposed to be built at DESY by 2010. He has been involved in the OPAL experiment at the Large Electron-Positron storage ring of the European Laboratory for Particle Physics.

Covert trade in toxic vetch continues

Sir — The Commentary “A mess of red pottage” by two of us¹ exposed the export from Australia of seeds from toxic common vetch (*Vicia sativa*) as a cheap substitute for the edible red lentil (*Lens culinaris*). Despite curbs to this trade in the wake of our article, the problem has recurred.

Dietary cyanoalanine neurotoxins from vetch accumulate in the brains and livers of pigs, rats and poultry. Effects on sulphur metabolism result in urinary excretion of cystathionine and accumulation of the non-functional glutathione analogue γ -glutamyl- β -cyanoalanyl-glycine. E. G. Brown² pointed out that the genetic predisposition towards favism in Mediterranean communities makes this doubly unfortunate, because common vetch contains the favism toxin vicine.

After our Commentary was published, India and Egypt banned, and Saudi Arabia restricted, imports of vetch. The *Victorian Weekly Times* published articles entitled “Scientists cripple vetch industry” and “Vetch now unfit for humans to eat”. The A\$18 million (US\$11.7 million) market collapsed.

But last year we were alerted by the Sri Lankan Health Department of the problem’s

re-emergence. Unlabelled bags of split red vetch (240 tonnes) had been exported from Adelaide via Johor in Malaysia to Colombo. On arrival, the container documentation had changed from “red split vetch” to “red split lentils”. This event was extensively reported in the Sri Lankan *Daily News*³, but was ignored by the international media.

In April 1999, a report on Australian ABC radio⁴ gave details of the multi-thousand tonne vetch/lentil substitution racket. The export price (A\$340–400 per tonne) leaves no doubt that the bulk is sold for human consumption. Exports (in tonnes) from South Australia for the first four months of 1999 include: split vetch to Yemen (22) and United Arab Emirates (194); and ‘whole vetch’ to Argentina (10), Bangladesh (989), Belgium (187), Germany (63), Italy (149), Japan (120), Mexico (40), The Netherlands (124), Pakistan (903), The Philippines (22), Portugal (106), South Africa (42) and Taiwan (86).

From these data, and the sale of unlabelled split red vetch in the markets of Pakistan and Bangladesh, we conclude that either it is being split in the importing country or it is being split in Australia and

exported labelled as whole vetch.

When cooked (without leaching) in mistake for red lentils, 1,000 tonnes of vetch gives ten million 100-g platefuls, each containing 0.5 g of cyanoalanine toxins. The harmful effects of vetch consumption in animals are known, but the effects on humans are not. Most at risk are malnourished vegetarian societies with sulphur-deficient diets.

To protect a valuable and expanding lentil industry, the Australian government moved swiftly (7 April 1999) to declare both vetch and lentils as prescribed grains. This means that inspection and phytosanitary certification are mandatory before export. Unfortunately, the financial rewards to exporters and importers are still too high to eliminate this dishonest trade.

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**Visiting scientist*

1. Tate, M. E. & Enneking, D. *Nature* **359**, 357–358 (1992).

2. Brown, E. G. *Nature* **360**, 9 (1992).

3. <http://www.lanka.net/lakehouse/archive.html> (8 October 1998).

4. <http://www.abc.net.au/rn/talks/8.30/helthrtpt/stories/s22429.htm>

Valuable biodiversity data under threat

Sir — Your report¹ on the move by the Burke Museum of Natural History and Culture to become independent of the University of Washington, Seattle, because of a lack of financial support from the university, illustrates one of the threats facing university biodiversity collections.

The Burke museum has the advantage of size to make plausible its becoming an independent institution. But curators of smaller, yet important, collections often face a similar financial crunch that cannot be solved by becoming independent. They have the option of minimal management of the collections, sometimes through out-of-pocket support, or of allowing the collections to become orphaned.

If orphaned collections are not transferred to an institution interested in their care, the result is a tragic, irreplaceable loss of biodiversity data. That is certain to hasten the decline of university training in systematic biology, which depends on collections. These and other factors contribute to what is aptly called “the crumbling infrastructure of biodiversity”².

It is to be hoped that universities will recognize that their biodiversity collections

are as important a component of the infrastructure for biological sciences as electron microscopes and ultracentrifuges.

Angelo Capparella

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1. Dalton, R. *Nature* **399**, 189 (1999).

2. Winker, K. *Cons. Biol.* **10**, 703 (1996).

Global e-journal must take radical approach

Sir — Much nonsense has been written about the ‘E-Biomed’ initiative to launch a global website for much of the biomedical literature, announced by Harold Varmus, director of the US National Institutes of Health (NIH). If it were the case that the present system had served science well for 300 years, for example, the outrage over the present state of scientific publishing felt in some quarters would not exist (see *Nature* **398**, 735 & **399**, 292; 1999).

Happily, dialogue has been under way worldwide for some time, involving people from various disciplines, to investigate how electronic publishing can reach the broadest possible audience. Some new model will emerge from this process. Harmonization of goals and means is difficult, and benefits

greatly from the involvement of a broad pool of perspectives and contributions. In this setting, the Varmus initiative is welcome, as are the critical evaluations to which the proposal will be subject.

Some of the valid concerns are the preservation and organization of materials; cataloguing; provenance (whether to peer review); copyright; and economic impact (including access in less developed countries). To advance the ‘E-Biomed’ proposal, I suggest that a moderated listserver is set up to initiate documentation and development of the larger community of interest. The NIH would be an appropriate host. These groups should be encouraged to participate: authors and researchers; archivists and librarians; Internet specialists; publishers and professional organizations; and readers.

I am initially mistrustful of what appears to be (largely) an electronic replication of existing methods of publication. One possibility is that ‘E-Biomed’ could host papers vetted by ‘alternative’ editorial boards, whose composition would be publicly stated, and whose editorial manifesto would be published, but whose membership would not be controlled by a central governing board.

Lance Sultzbaugh

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Charting the shores of nuclear stability

Neil Rowley

A fascinating challenge in nuclear structure physics is to find the maximum atomic number Z – the number of protons – for which a relatively stable nucleus can exist. The question is, just how many stable elements is it possible to make?

The creation of three new elements this year, $Z = 114$ at the Joint Institute for Nuclear Research in Dubna, Russia¹, and $Z = 118$ (with $Z = 116$ following on from radioactive decay of 118) at the Lawrence Berkeley National Laboratory in the United States², are momentous events in the field of superheavy-element production. The new element 114 was created at Dubna through nuclear fusion, by bombarding a heavy isotope of plutonium with a beam of ^{48}Ca ions. A follow-up experiment at Dubna, which verifies the properties of a second isotope predicted from those of the first, is reported on page 242 of this issue³. All the new results confirm the possibility of the fabled 'island of stability' of superheavy nuclei with roughly 114 protons and 184 neutrons. Until now, all artificial superheavy nuclei have decayed within milliseconds of formation, whereas nuclei near the island of stability are expected to last for years (see Box 1, overleaf).

The stable nuclei existing in nature, or at least having lifetimes comparable with the age of the Universe, are shown by the small black squares in Fig. 1. Many other nuclei have been created artificially — those for

which mass measurements are available are indicated by grey squares. These nuclei decay through the weak interaction (that is, the β -decay of a proton into a neutron, or vice versa); or by α -particle decay (emission of a ^4He nucleus, reducing Z , the number of protons, by 2); or by proton emission; or by spontaneous fission (breaking into two fragments of comparable mass).

To bridge the gap between the stable or long-lived nuclides and the putative island of stability, one must forge a compound nucleus by fusing two nuclei of lesser mass and charge. Three heavy elements, $Z = 110, 111$ and 112 , produced at the Gesellschaft für Schwerionenforschung in Germany in 1994 and 1996, were created by fusing a target nucleus, such as ^{208}Pb (which has 82 protons and 126 neutrons), with an appropriate lighter projectile⁴. But the nuclei produced by this method fall some way short of the island (Fig. 2). The idea behind the Dubna experiments is to use a neutron-rich projectile of calcium, ^{48}Ca (which has $Z = 20$), on targets of plutonium ^{244}Pu and ^{242}Pu (which both have $Z = 94$) in order to arrive at the desired element 114, while getting in much closer to the island in terms of the number of neutrons, N .

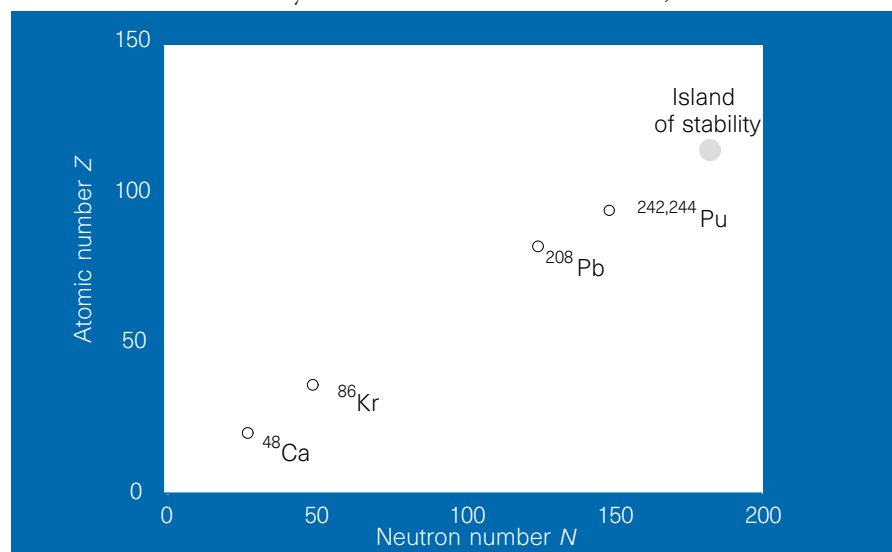


Figure 1 Chart of the nuclei. The black squares represent the stable or very long-lived nuclei in relation to their neutron number N and proton number Z . The grey squares represent artificially created nuclei for which mass measurements exist. The grey circle shows the area of the island of stability centred on $Z = 114$ and $N = 184$, and the open circles highlight the targets and projectiles used in the recent experiments^{1–3}, which created new superheavy elements.

The use of such exotic materials is rather difficult, because ^{48}Ca is extremely rare in nature and requires painstaking isotope separation to obtain sufficient quantities for the very intense beams necessary for these experiments. In addition, the plutonium target is highly toxic and radioactive and demands considerable care in handling. Even then, with ^{48}Ca ($N = 28$) and ^{244}Pu ($N = 150$), the combined neutron number, 178, still falls short of the desired 184, but this is probably the best one can do with stable or long-lived targets and projectiles. (Note that ^{242}Pu and ^{244}Pu have lifetimes measured in thousands and millions of years respectively, but the lifetime of ^{246}Pu is only 10.8 days, making it useless as a target.)

To fuse the target and projectile, they must be launched together with a relatively high kinetic energy to overcome the repulsive electrostatic or Coulomb energy between their positive charges. Below this Coulomb barrier the probability of fusion drops drastically, and even at this energy the compound nucleus is formed at a temperature at which such a heavy system is very unlikely to survive fission. Although the fusion probability increases gradually above the Coulomb barrier, the survival probability falls off steeply. The tiny survival probability is reflected in the experimental data. For example, in the first Dubna experiment, only one event out of 5.2×10^{18} incident calcium ions (over a 40-day period) corresponded to a compound nucleus that survived to give a nucleus with $Z = 114$! It did so by a chance rapid cooling below the temperature where fission is inevitable by evaporating three neutrons, leading to a final 'evaporation residue' with only 175 neutrons. So although the original compound nucleus is parachuted over the beach of the island of stability,

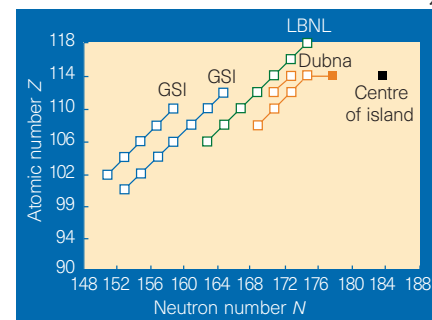


Figure 2 Decay chains of various superheavy nuclei. Fusion experiments create heavy evaporation residues, which lie at the top of each chain, and the connecting lines represent subsequent α -decays ($\Delta N = \Delta Z = -2$). The black square is the centre of the island of stability and the grey square the compound nucleus created in the initial experiment at the Joint Institute for Nuclear Research in Dubna, Russia (that is, before neutron evaporation). GSI, Gesellschaft für Schwerionenforschung, Germany; LBNL, Lawrence Berkeley National Laboratory, USA.

NATURE

100 YEARS AGO

This afternoon, as I was walking into Lickey Village from King's Norton, I came across innumerable frogs. They lined the hedges and covered the road so thickly that I had to walk on tiptoe. I thus proceeded quite 400 yards, where the phenomenon ended as sharply defined as it had begun. Nowhere else along the road was a frog to be seen. I was particularly astonished, as I knew the nearest water to be the Little Reservoir – quite $\frac{1}{2}$ mile away. The frogs were about ten days old, very small. A cottage stood about 300 yards from the beginning of this swarm. Upon enquiry I ascertained that the frogs had thus congregated since noon on Monday, that they had literally besieged the house, jumping all over the ground-floor rooms, that the garden and its paths were full of them. The present occupants had lived there $4\frac{1}{2}$ years, but had never experienced anything like this. They have sometimes seen a few frogs cross the road in wet weather. They are now occupied with brushing them out of doors. Can any of your readers explain the cause of this extraordinary spectacle? From *Nature* 13 July 1899.

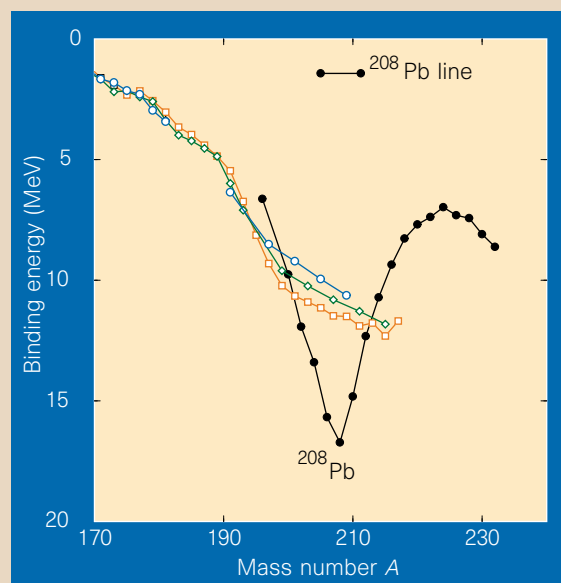
50 YEARS AGO

The centre of the Ciba Foundation at 41 Portland Place, London, W.1, was opened by Sir Henry Dale on June 22. This foundation is, we believe, unique in conception and purpose. The ideal of free flow and inter-communication of results of research in medicine and chemistry is very far from realization. The impediments normally present in the way of full collaboration are those of professional jealousy, indifference between academic and commercial spheres, trade competition and secrecy, languages, national prejudices, political and economical factors, distance, and so on. Such impediments become insurmountable barriers in the ruthlessness of war and when national fears exact secrecy. The directors of the well-known firm of Ciba, of Basle, ... recognized the potential vigour of research when international cross-fertilization could take place, as exemplified in Anglo-American scientific co-operation. ... It was agreed to endow an institution to encourage, so far as possible, personal meetings of research workers from all over the world. From *Nature* 16 July 1949.

Box 1: The island of stability

The stability of a nucleus is directly related to its binding energy (that is, the difference between its mass and that of its component protons and neutrons). For all but the lightest nuclei, this binding energy can be described on average by the relatively simple classical model of a 'charged-liquid drop'. But, as one would expect for such small systems, quantum mechanics can give strong local variations determined by the energies of the individual orbitals that the nucleons can occupy.

Quantum mechanics gives rise to energy levels for the individual neutrons and protons, which have 'shell gaps' rather than being evenly distributed. A 'magic number' of nucleons arises when they fill up the levels to just below the shell gap. The compression of the energy levels that such nucleons occupy can lead to a significant increase in the total binding energy, and hence stability. This 'shell effect' persists over a range of nucleon numbers around the magic numbers, and is obviously more important if both neutron and proton numbers are close to magic numbers. See, for example, the



graph, where the filled circles represent isotopes of constant $N-Z=44$, with a maximum binding energy for the nucleus ^{208}Pb , which has two magic numbers ($Z=82$ and $N=126$).

Results of many theoretical calculations indicate that the next magic numbers should occur for $Z=114$ and $N=184$, leading to the predicted island of stability for nuclei in the range $Z=104$ to 124 , with appropriate numbers of neutrons. The graph shows the variation in binding energy for nuclei in the vicinity of ^{208}Pb , where the nuclear masses are well known. The nuclei shown are the same relative distance (in terms of N and Z) from

^{208}Pb as the new superheavy nuclei are from the centre of the island of stability. If we had accurate masses for these superheavy elements then the various plots would correspond to isotopes created in the long Dubna chain (open squares), the short Dubna chain (open diamonds) and the new LBNL chain (open circles). The shell effect is clearly seen to persist in the neighbourhood of the doubly magic ^{208}Pb , suggesting a similar effect near the island of stability. The precise values of the new magic numbers depend on details of the basic nucleon-nucleon interaction, making experimental data in this region very valuable. **N.R.**

the prevailing winds of neutron evaporation blow the final system out into the shallow waters off the coast.

The real *tour de force* in such experiments is, of course, the detection of the superheavy needle in the haystack of other events. This is made possible with the help of a gas-filled mass separator that detects the recoiling evaporation residues and ensuing chain of α -decays, which must all occur at the same location in the separator to confirm the existence of a superheavy element. Ironically, this technique would never show up a gen-

uinely stable nucleus if one were created! (Although even the nucleus at the centre of the island is not predicted to be totally stable but rather to have a significantly lengthened lifetime.) The reward of such an elaborate and time-consuming experiment is the production of an isotope of $Z=114$ with the amazingly long lifetime of about 30 seconds, orders of magnitude greater than nuclei produced earlier in this vicinity. This is a clear indication that the island is near.

The second Dubna experiment uses the lighter isotope of plutonium and is designed

to give a nucleus with 114 protons and 173 neutrons, which falls into slightly deeper water off the island. So far there have been two events. But the beauty of this experiment is that the decay properties of the new isotope could be calculated from the observed properties of the first isotope of element 114. The prediction of a single α -decay followed by spontaneous fission is confirmed convincingly³.

In the latest LBNL experiment², the previous philosophy of using a ²⁰⁸Pb target was adopted. This nuclide has a closed shell of neutrons ($N = 126$) and of protons ($Z = 82$) producing an unusually stable nucleus (see Box 1). The extra binding energy of this double-closed-shell system leads to a cooler compound nucleus that needs to evaporate only one neutron to survive fission. A projectile with 36 protons (⁸⁶Kr) enabled the group to leapfrog element 114 and reach $Z = 118$ (seen in three events), which then decays to $Z = 116$, 114 and so on. In terms of the ques-

tion "Just how many stable elements is it possible to make?", this increases the maximum atomic number by a further four units. But, for the isotope of $Z = 114$ observed in the long Dubna decay chain (Fig. 2), the lifetime is over four orders of magnitude greater than that for the $Z = 114$ isotope in the LBNL chain. Similar comparisons hold for $Z = 112$ and $Z = 110$. Even the 114 isotope in the short Dubna decay chain (Fig. 2) has a lifetime enhanced by over three orders of magnitude. So, in terms of reaching the island of stability, the Dubna experiments appear so far to be the closest. □

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Behavioural ecology

Electrifying diversity

Michael J. Ryan

Sexual selection often favours signals that are conspicuous; the more conspicuous the signal, the more attractive the signaller is to mates¹. But conspicuousness incurs a cost because predators can eavesdrop; the more conspicuous the signal, the more risky it is. So natural selection generated by predation is often viewed as a constraining force in signal evolution. In the face of predation, for example, crickets evolve non-calling strategies, guppies evolve dull colours, and túngara frogs produce less conspicuous calls¹. In a study described on page 254 of this issue², however, Philip Stoddard argues that predation by electrolocating predators on gymnotiform electric fish has been a creative rather than a constraining force on the evolution of electric-organ discharges (EODs).

There are more than 100 species of gymnotiforms, or knife fish, in the fresh waters of Central and South America. These animals are nocturnal, and often live in murky waters where visual communication would be difficult even in the daytime. But poor visibility has little effect on them, as they rely on their ability to produce and detect weak electrical fields for sensing their environment as well as for communicating with one another. The EODs, however, make the fish vulnerable to electropredators such as electric eels and catfish.

It seems that the ancestral EOD was an intermittent monophasic pulse. But many gymnotiforms produce a biphasic EOD. What forces might favour the evolution of such a signal? Stoddard addresses three alternative hypotheses: electrolocation, sexual

selection and predator avoidance. He argues that predator avoidance was the initial force favouring the evolution of the complex EODs, although sexual selection might have then been responsible for the subsequent

sexual dimorphism in the EOD of some gymnotiforms.

Stoddard presents both correlational and experimental evidence that predation can favour the use of biphasic signals. One can change a monophasic EOD to a biphasic one by adding a negative-going second phase, which shifts the frequency upwards (Fig. 1). All electrolocating fish have ampullary organs, which are extremely sensitive to low frequencies and excel at detecting the weak electric fields of prey. But these receptors are less sensitive to the higher frequencies of the biphasic EODs that many gymnotiforms use in communication (Fig. 1). Gymnotiforms also evolved a second set of electroreceptors, tuberous organs, which are less sensitive than ampullary organs but are tuned to the higher frequency of the EOD. So both the signal and the receiver in the gymnotiform communication system operate in a frequency range above that to which ampullary organs are most sensitive. It would seem that such a frequency shift would reduce predation risk.

To test this hypothesis, Stoddard and his graduate student Marina Olman trained an electric eel, a predator, to respond to electrical discharges; the reward was associated with approach to either a monophasic or biphasic EOD. The predator was more likely to approach the monophasic pulse. These results suggest that there is an advantage to producing biphasic pulses, but do not

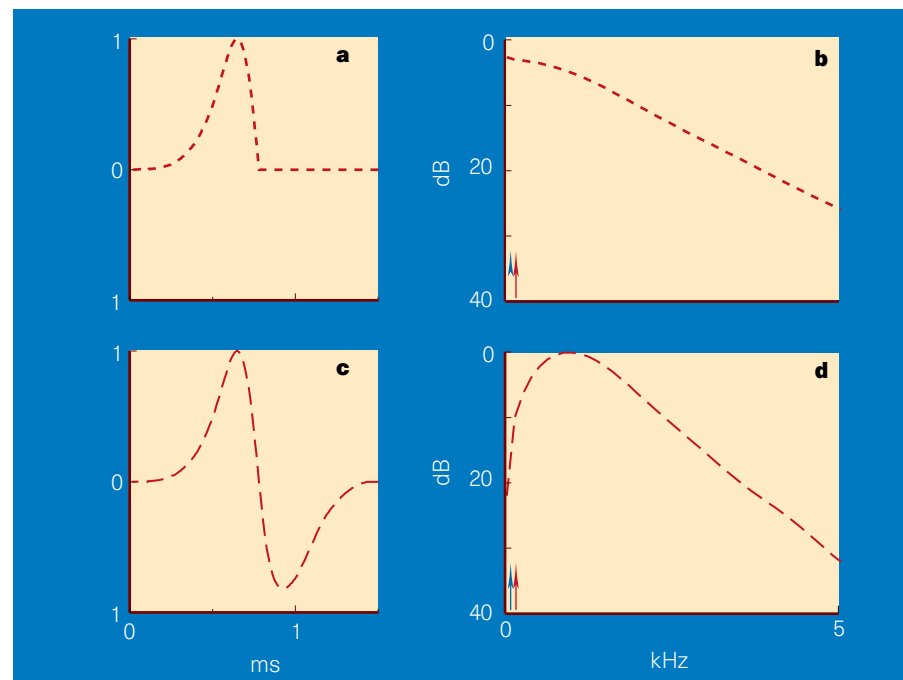


Figure 1 Signalling in electric fish. The waveform of a monophasic (a) and biphasic (c) electric-organ discharge (EOD) with the corresponding power spectra (monophasic, b; biphasic, d). The arrows show the sensitivity of two types of electroreceptive predators, catfish at about 8 Hz (blue) and gymnotiforms at about 30 Hz (red). The biphasic EOD is from *Brachyopomus pinnicaudatus*, gymnotiform prey; the monophasic waveform is that same waveform with the negative component deleted. The figures are modified from Figs 3c and 3d of Stoddard², who argues that predation has favoured an increase in the complexity of EODs because the biphasic signals are less detectable by predators than are the monophasic signals.

explain why three species of gymnotiform still produce monophasic EODs. These, Stoddard argues, might be the exceptions that prove the rule. Of the three monophasic species, one is an electropredator, one lives in an area devoid of electropredators, and one produces an EOD which resembles that of an electric eel.

This study describes a convincing way in which predation may have promoted rather than constrained signal diversity. But it is not a unique case, as predation is known to have had similar effects in other systems. It caused moths to evolve ears to detect, and signals to deter, bat predation; these traits were then co-opted for communication, resulting in males that communicate their presence to females with ultrasonics (in some species, males and females even conduct an ultrasonic duet)³. Also, it has been suggested that offspring might evolve signals that are more conspicuous to predators in order to manipulate parental behaviour⁴; bird nestlings giving loud, conspicuous begging calls, and children holding their breath in parental defiance, are two examples.

Stoddard's study gives us a glimpse into the complicated world of signal evolution of one system; a more general understanding, however, is far off. Consider Zuk and Kolluru's⁵ review of the predation costs of sexual signals. They pointed out that there are many more examples of predators attracted to signals in the acoustic mode than in the visual mode (electrical communication is more similar to acoustic than visual communication). This bias is probably because it is easier to study acoustic than visual communication. Nevertheless, it is crucial to know how predator effects on communication systems might vary among sensory modalities.

For example, is it as likely that potential prey can evolve electrical (say, biphasic pulses) or acoustic (say ultra- or subsonic) signals out of the range of their predators than it is for prey to evolve visual signals (say in the ultraviolet) to escape predators in that modality? One part of the answer might depend on the lability of signals in a given modality. Another must derive from the predator's receiving systems. Do the demands of communication on a sensory system constrain the uses of that system in other tasks, and does this vary among sensory modalities? For example, does tuning an electroreceptive or auditory system to one type of signal, a weak electric field or an echolocation call, constrain this system from being used to locate prey making very different types of sounds? It might be so in electric eels, but appears not to be in frog-eating bats⁶.

Also, more generally, we can ask how constrained different sensory systems are in their ability to evolve. Can we compare the

changes in the inner ear of a vertebrate, needed to allow that animal access to ultrasonic emissions made by its prey, to changes in the retina of the same animal that would give it access to ultraviolet signals of different prey? Probably not. Only studies of the entire biology of communication systems can allow an appreciation of their diversity — an argument that provides strong support for the kind of integrative approach taken by Stoddard. □

Signal transduction

Neither straight nor narrow

Mark Peifer

Back when I was a boy, things were simpler. Web browsers fancied spiders, cell phones were used by prisoners to call their lawyers, and signal transduction — the mechanism by which information is transferred from the surface of a cell to its nucleus — proceeded through a linear series of simple steps. But times have changed, and five papers in *Nature*^{1–5} (the latest of which are found on pages 271, 276 and 281 of this issue) provide a dramatic example of this. The discovery of new components and connections is converting the stepwise, linear pathways of information transfer into increasingly complex networks.

Most of our cells do not act in isolation — rather, they respond to signals from their neighbours. Much of the cellular machinery is devoted to receiving these signals and then transducing them, relaying information to the components that mediate the cell's response. In the simplest view, these signal-transduction pathways are envisaged as linear wiring diagrams, like telephone lines that link individual customers to the phone company.

The decisions that cells make are dominated by just a few families of cell–cell signals. Among these are members of the Wingless signal-transduction pathway, inappropriate activation of which contributes to human cancers. (On a lighter note, this pathway may also offer a cure for baldness⁶.) The genes required for Wingless signalling were first identified in the fruitfly *Drosophila melanogaster* (and, for simplicity, only the *Drosophila* names of components in the pathway are mentioned here). The pathway initially seemed simple and linear (Fig. 1; and reviewed in ref. 7). A protein called Armadillo is the key regulated component. Normally, Armadillo is unstable inside cells because it is targeted for destruction by a kinase known as Zeste-white3 (Zw3). This kinase is somehow counteracted by another protein, Dishevelled, which is activated when Wingless binds at the cell surface. The receptors for Wingless signals are members of the Frizzled

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Figure 1 Our humble beginnings — early ideas about the Wingless signalling pathway. Initially this pathway was thought to be a simple, linear cascade. Binding of Wingless to its receptor leads to activation of Dishevelled, which counteracts the repressive effects of the Zeste-white3 (Zw3) kinase and allows expression of Armadillo. After crossing into the nucleus, Armadillo can then activate Wingless-responsive genes.

protein family, distant relatives of guanine-nucleotide-binding (G)-protein coupled receptors. So, when Wingless binds to Frizzled receptors, Dishevelled is activated, somehow counteracting Zw3 and stabilizing Armadillo. Armadillo then enters the nucleus, where it interacts with transcription factors and activates Wingless-responsive genes.

But this simple, linear picture has changed dramatically — additional regulatory inputs have been discovered at each level in the pathway. There are, for example, many receptors in the Frizzled family, as well as many Wingless ligands, and we are only just beginning to discover how these proteins can be mixed and matched. Moreover, secreted antagonists of Wingless (a subset of which are secreted analogues of Frizzled receptors), negatively regulate signalling (reviewed in ref. 7). The papers by Tsuda *et al.*² and Lin and Perrimon³ further complicate the issue, because they suggest that the Wingless receptor is, in fact, a protein complex, with

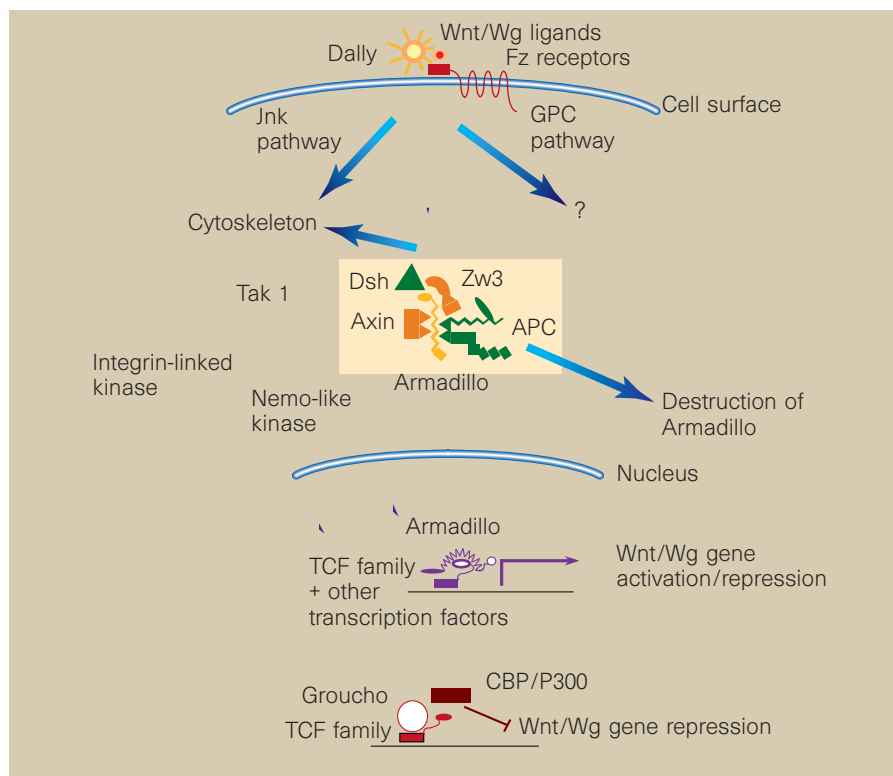


Figure 2 Current picture of the Wingless signalling pathway. The results from a slew of papers, including three in this issue^{1–3}, reveal a much more complicated pathway than that depicted in Fig. 1. Although Armadillo is still a key regulated component, other events may result from a number of ligands (members of the Wingless family; Wnt/Wg) binding to a number of different receptors (Frizzled; Fz). These include direct effects on the cytoskeleton, possibly through the Jnk pathway, and other effects mediated by a distinct G-protein-coupled (GPC) signalling pathway.

Frizzled receptors using a heparin-sulphate-modified proteoglycan called Dally as a co-receptor (Fig. 2).

Destruction of Armadillo is triggered by two multiprotein complexes (reviewed in ref. 8). The complicated interactions between the proteins in multiprotein complexes make it difficult to think about what happens there in linear terms. The Zw3 kinase was the first component of the destruction machinery to be identified. The second, found in the same complex, was the tumour-suppressor protein adenomatous polyposis coli (APC). Loss of APC function — which underlies both familial and sporadic colon cancer — leads to accumulation of the vertebrate homologue of Armadillo (β -catenin) and activation of downstream target genes such as *c-myc* and *cyclin D*, which, presumably, drive cell proliferation.

But the picture is even more complicated because Dishevelled, which is essential for activating Armadillo, can also reside in the multiprotein complex^{9–11}. So, the 'destruction' complex may activate Armadillo as well as repressing it. When repressing Armadillo, the destruction complex helps Zw3 to phosphorylate it. Phosphorylated Armadillo then binds to the second multiprotein complex, which is built around an F-box protein called Slimb (reviewed in ref. 12). Slimb and its protein partners add a ubiquitin tag to

Armadillo, targeting it for destruction by the proteasome.

So, activation of the Wingless receptor regulates the stability of Armadillo by inactivating the destruction complex. Cytoplasmic Armadillo accumulates, and then enters the nucleus where it forms a complex with DNA-binding proteins of the T-cell factor (TCF)/lymphoid enhancer factor family (reviewed in ref. 13). This complex activates Wingless-responsive genes, and Armadillo is thought to be directly involved, contributing a transcriptional-activation domain. But here again the circuitry is more complex than anticipated. In the absence of Armadillo, TCF proteins repress Wingless target genes; binding of Armadillo antagonizes this repression. Moreover, other transcription factors (such as the zinc-finger protein Teashirt¹⁴) may regulate Wingless signalling by binding to Armadillo. Payre *et al.*¹ now reveal a further variation on this theme. The authors find that the Armadillo/*Drosophila* TCF complex not only activates genes, but that it represses them too. By preventing expression of a protein called Shavenbaby, which is a transcriptional regulator of cell fate and the cytoskeleton in the *Drosophila* embryonic epidermis, Armadillo/TCF causes the correct cuticular pattern to be formed.

But any hopes of an essentially linear —

albeit complex — pathway have been dashed by observations suggesting a branched pathway with many inputs and outputs. The first evidence for this came from the finding that different Frizzled receptors signal through distinct signal-transduction pathways. As well as the Wingless pathway, some Frizzled receptors seem to act through G proteins, Ca^{2+} and protein kinase C, whereas others may activate the Jnk signalling pathway (reviewed in ref. 7). Moreover, Frizzled signalling can target cellular processes other than gene expression. The original Frizzled receptor was identified because of its effects on the actin cytoskeleton (a process known as tissue polarity), and Wingless signals also affect the microtubule cytoskeleton, polarizing the spindle during asymmetric cell divisions in nematodes and flies (reviewed in ref. 15). Wingless acts directly on the mitotic spindle and does not need to activate gene expression to do so¹⁶, suggesting that its effects occur through a branch in the pathway at the kinase Zw3 (Fig. 2).

As well as these alternative outputs there also may be independent inputs. Meneghini *et al.*⁴, Ishitani *et al.*⁵ and Rocheleau *et al.*¹⁷ provide evidence for a second pathway that converges on β -catenin/TCF in worm and mammalian cells. These authors show that a divergent mitogen-activated protein kinase pathway involving Tak1 and Nemo-like kinases is required for Wingless signalling in the nematode *Caenorhabditis elegans*. They go on to show that Nemo-like kinases can phosphorylate both WRM-1 (a relative of Armadillo) and POP-1 (a relative of TCF). In so doing, these kinases can affect both DNA binding and nuclear localization. It is not clear whether this pathway is a branch downstream of a Frizzled receptor (like Frizzled, *Drosophila* Nemo is involved in tissue polarity), or whether it is an independent input into β -catenin/TCF activity. Another possible independent input may come through integrin-linked kinase, which is a component of focal adhesions, junctions that join cells to the extracellular matrix. Activation of this kinase stabilizes β -catenin and can activate expression of a Wingless-responsive reporter gene¹⁸.

Despite our desire for cells to think in linear terms, they refuse to do so. Evolution has cobbled together remarkably elaborate machines that allow cells to respond to signals from their environment. Those of us wedded to the idea of signal-transduction pathways as the shortest route from the membrane to the nucleus will have to adjust to the new millennium, in which intricate networks integrate many inputs to generate the complex output that is cell behaviour. □

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Quantum optics

A box for a single photon

Philippe Grangier

The controlled manipulation of individual quantum objects, such as atoms, ions or photons, has developed considerably in recent years. A 'billiard ball' approach to individual atoms has been popularized by the fantastic landscapes created and explored using atomic force microscopy techniques, which exploit the repulsive force between microscope tip and atomic sample. But many experiments about the wave nature of matter, or about the particle nature of light, remind us that the fundamental nature of microscopic objects obeys the laws of quantum mechanics. A single photon provides the most striking evidence that a quantum particle is something much more elusive than a billiard ball. A new experiment reported on page 239 of this issue¹ demonstrates how to store a single photon, and, more importantly, how to watch it repeatedly.

As far as the position and momentum of particles are concerned, Heisenberg's well-known 'uncertainty relation' claims that both cannot be known simultaneously with perfect accuracy. A related idea is that a precise measurement in the microscopic world is not possible without introducing a perturbation, or 'back action', inherent in all measurements. For example, a very precise measurement of a particle's position will greatly disturb the particle's momentum and vice versa. This can be generalized to any pair of 'non-compatible' physical quantities, represented in quantum language as operators A and B . Although the precision in a single measurement of A is not restricted, the large fluctuations induced in B when measuring A may eventually couple back to A , which will then also be perturbed, making it difficult to perform repeated or continuous measurements. In response to this problem the concept of 'quantum non-demolition' (QND) measurements was introduced in the 1970s, in which a measurement strategy is chosen that evades the undesirable back action². The key issue is to devise a measurement scheme so that the back-action 'noise' is kept entirely within unwanted observables. The quantity of interest then remains uncontaminated by

the measurement process, allowing repeated measurements to be performed with arbitrarily high accuracy.

When the quantity to be monitored is the number of light particles—that is, the number of photons—a QND measurement is the best way to proceed. In the optical domain, QND experiments have already been used to explore the ultimate limit on the non-destructive extraction of information encoded in a laser beam³. The relevant Heisenberg relation is the so-called number–phase inequality, which sets a lower limit on the product of the dispersion ΔN (in the number of photons, N) and the dispersion $\Delta\phi$ (in the phase of the light wave, ϕ), such that $\Delta N \Delta\phi \geq 1/2$. In optical QND experiments, N is a very large number (so that $N \gg \Delta N \gg 1$) and $\Delta\phi \ll 1$. Although it is

not possible to resolve individual photon numbers, it can be shown³ that the sensitivity of the measurement enters the quantum regime when ΔN is smaller than the 'shot-noise limit', $N^{1/2}$. To go beyond this 'large number' situation, experimentalists have to achieve single-photon resolution (that is, $\Delta N < 1$), allowing them to watch individual photons. Such experiments require extremely strong coupling between the measured 'signal' light field and another quantum system, usually called the 'meter', which reads out the number of photons without perturbing the signal.

This challenge has been met by a group working at the Ecole Normale Supérieure (ENS) in Paris¹, using the experimental techniques of cavity quantum electrodynamics (cavity QED). Here the 'signal' system is a microwave field confined within a niobium cavity, which is cooled by liquid helium to make it superconducting and thereby increasing the storage time of photons in the cavity up to one millisecond. The 'meter' system is made of rubidium atoms that cross the cavity one at a time, and therefore pick up information about the light field (see Fig. 1). Ideally, according to the basic requirement for QND measurements, there should be no exchange of energy between the signal and meter. This can be obtained by having the frequency of light different from that of the rubidium atomic transition. In this 'non-resonant' situation the exchange of information is purely dispersive, that is, the atomic wavefunction only picks up a phase shift,

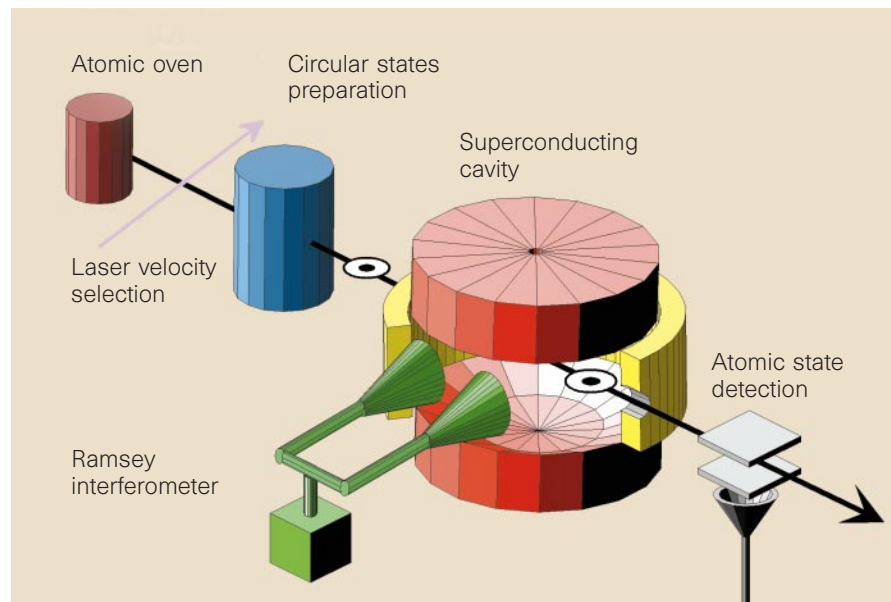


Figure 1 How to detect a single photon repeatedly without destroying it. The photons used by Nogues *et al.*¹ are stored in the cavity (red) in the centre of the figure. The ring (yellow) between the two mirrors is cut open to reveal the inside of the 'photon-box'. Using lasers, rubidium atoms travelling along the main arrow are prepared in an appropriate state for interacting with the cavity field. These atoms (in 'circular' Rydberg states) have a very long lifetime, well suited for the experiment. The velocity selection and pulsed excitation scheme allows the position of each atom to be known. The Ramsey interferometer (green) is coupled to the inside of the photon box through holes in the ring (not shown). Combined with the detector (grey) it measures the phase of the atomic wavefunction. The value of this phase tells you whether the cavity contains zero or one photon.

which is proportional to the number of photons in the cavity. Such a scheme was proposed a few years ago by the ENS group⁴, but it has not been implemented yet, because the fact that the atom–field interaction is non-resonant makes it very weak. In the present experiment¹, a different trick is used: the interaction is fully resonant and thus much larger. An energy exchange does occur, but the parameters are chosen so that this energy exchange is fully reversible.

To understand how this exchange happens, we need to look briefly at concepts developed in the field of cavity QED. To cut a long story short, cavity QED uses optical or microwave resonators designed in such a way that the ‘electric field per photon’ is extremely large. Consequently, the coupling rate between an atom and the field is also very large, much larger than the dissipative effects caused by spontaneous emission from the atom, or the electric field leaking out of the cavity. So when the atom is within the cavity, the system can be described by only considering the coherent coupling between the atom and the cavity under various conditions. If there is no photon in the cavity, nothing happens; if there is one photon in the cavity, it is coherently absorbed and re-emitted by the atom before it leaves the cavity. In the latter case, the net energy exchange is zero, but it can be shown that because of this ‘Rabi cycle’ a phase shift occurs in the atomic wavefunction. Finally, if there is more than one photon, more Rabi cycles will occur, but this case can be ignored by the experimenters.

If one considers only the cases where the cavity is initially in an arbitrary quantum superposition, or mixture, of zero-photon and one-photon states, it is clear that this set-up will behave in a QND way. The phase information is extracted from the ‘meter’ atom using an interference effect, which transforms the phase shift into a detectable change in the atomic level. In fact, several schemes have been used by the ENS team¹: either a first atom is used to deposit one photon within the cavity and a second atom detects it; or both atoms perform two successive measurements of a weak initial field, repeatedly allowing the experimenter to know whether the cavity contains zero photons or one photon.

The ENS format¹ cannot easily be generalized to higher photon numbers (this would require using the non-resonant interaction described above), but it is worth pointing out that the techniques that yield this single-photon QND measurement may also generate other interesting effects. The fact that one photon can significantly shift the atomic phase is the key to building a quantum logic gate, suggesting a route to quantum computing⁵. It can be expected that this experiment will open the way to many other ones, which will explore further the very peculiar rules

for writing in and reading out the information encoded in quantum objects. □

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Neurobiology

Monkeys play the odds

M. James Nichols and William T. Newsome

On a sunny, spring morning, an experienced fisherman furrows his brow and weighs the merits of one fishing hole against another further upstream. At both sites he sees promising signs, and a novice could not distinguish between the two. But the veteran knows that, on mornings like this, bigger fish bite more often upstream — and he selects the upstream spot.

Like the fisherman we all make countless day-to-day decisions — selecting restaurants, betting in office football pools, buying used cars, and so on. We base these decisions not just on current information, but also on accumulated experience and expectations about the likelihood and size of rewards. Experimental evidence indicates

that animals also shape their behaviour based on the expected size and probability of rewards. Indeed, economists^{1–4}, psychologists^{5–8} and ecological biologists^{9,10} place these critical variables at the centre of models about human and animal decision-making.

Platt and Glimcher (reporting on page 233 of this issue)¹¹ have now applied decision theory to combined behavioural and neurophysiological experiments in rhesus monkeys, providing new insights into how decision variables are represented in the central nervous system. Despite the importance of reward expectations in shaping behaviour, neurophysiologists have largely ignored them in studies of brain function (but see Gallistel⁵). Instead, they have adopted a framework in which particular patterns of

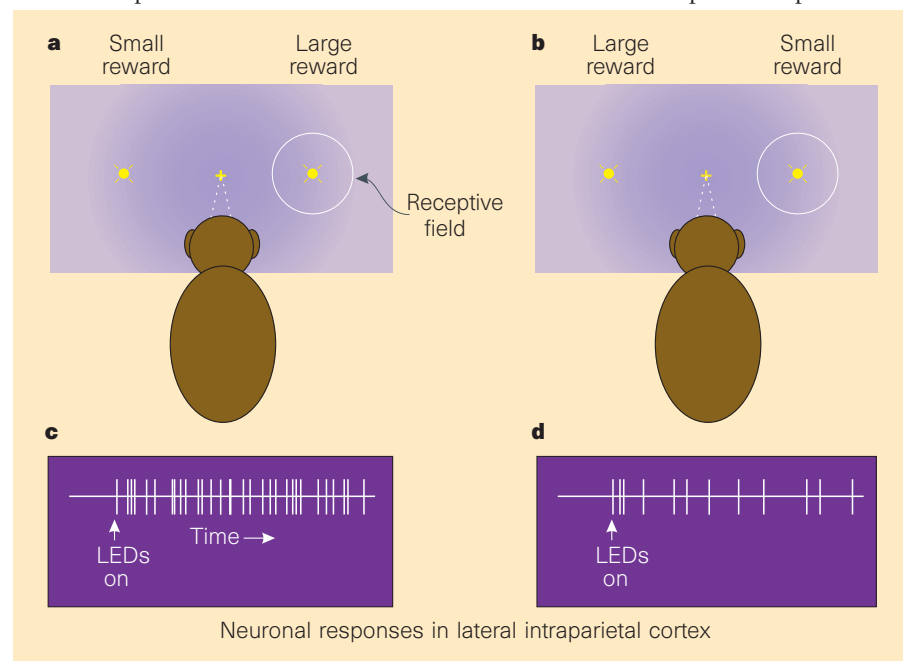


Figure 1 Assessing neural correlates of decision making. In a simple decision-making task devised by Platt and Glimcher¹¹, a monkey sits facing a viewing screen. On each trial, he first looks at a central fixation point (yellow cross). Then, two light-emitting diodes (LEDs; yellow circles) are illuminated at different locations on the viewing screen, one inside the receptive field (white circle) of the neuron under study, the other outside. After a delay, the fixation point is extinguished and the monkey receives a juice reward for looking to either of the two LEDs. **a**, In some blocks of trials, the LED within the receptive field is associated with a larger reward. **b**, In other blocks, the same LED is associated with a smaller reward. **c, d**, Schematized neuronal responses to the onset of the LED in the receptive field for the two reward conditions depicted in panels **a** and **b**. Vertical hatch marks indicate action potentials after illumination of the LEDs (vertical arrow). This hypothetical neuron discharges more vigorously for the same visual stimulus in its receptive field when the stimulus is associated with a larger juice reward.

sensory information are reflexively transformed into appropriate actions, with little regard for reward expectations or other variables. Although this simplified framework has been extremely powerful in early studies of the transformation from sensation to action, it cannot encompass the richness of the decision process.

In a key experiment (Fig. 1), Platt and Glimcher¹¹ placed a monkey in front of a viewing screen and repeatedly presented him with two identical visual stimuli — light-emitting diodes (LEDs). For each presentation of the two LEDs (called a 'trial'), the monkey was rewarded with a squirt of juice if he directed his gaze from an initial target (a yellow cross called a 'fixation point') to either of the two LEDs. In other words, the animal, like the fisherman, was required to choose between two visually indistinguishable alternatives. Then, in blocks of roughly 100 trials, the authors manipulated the size of the juice reward associated with each target. In one block of trials, for example, the reward associated with one LED might be twice the size of the reward for the other one (Fig. 1a). In the next block of trials, these reward sizes might be reversed (Fig. 1b). Platt and Glimcher found that, over several trials at the

beginning of each block, the monkey learned the reward contingencies and adjusted his target selection accordingly. Specifically, he preferentially selected one target over the other in proportion to the relative reward sizes for the two targets.

While the monkey performed this simple decision-making task, the authors recorded neural activity in the lateral intraparietal cortex (LIP)¹². This area receives inputs from several parts of the brain involved in processing visual information, and it projects directly to the areas that control eye movements. Neurons in LIP discharge action potentials in response to visual stimuli that lie in restricted regions of visual space (their 'receptive fields'). These neurons also discharge action potentials before and during gaze shifts to those visual stimuli. But neurons in LIP do not respond to stimuli that fall outside their receptive fields. Thus, LIP occupies an important intermediate stage in the transformation of visual signals into neural commands to move the eyes, and offers a point at which reward expectations could influence this transformation.

In each recording session, Platt and Glimcher placed one LED inside the receptive field (dashed circle in Fig. 1a and b) of the

neuron under study, and the other LED well outside it (although presumably within the receptive field of neurons in a different part of LIP). Consistent with previous results, when the two LEDs appeared, the one inside the receptive field elicited a response from the neuron. Furthermore, when the monkey shifted its gaze towards the LED in the receptive field, the neuron discharged before and during the eye movement, again consistent with previous findings. But the authors additionally found that when larger rewards were given for the LED in the receptive field than for the LED outside it (Fig. 1a), the neuron discharged more action potentials (vertical hatch marks in Fig. 1c) than when the same LED was associated with smaller rewards (Fig. 1d). So, given exactly the same visual display and exactly the same gaze shift, neurons in LIP respond differently depending on the animal's expectation of reward size — an expectation that is evident in the pattern of decisions.

Many questions remain. Where in the brain are different rewards evaluated? Where are they associated with particular sensory stimuli? Where are reward expectations calculated and stored? Which areas of the brain are involved in more complex decisions? Platt and Glimcher have focused on LIP, but these processes are probably distributed across many regions of the brain. Furthermore, although monkeys estimate reward size efficiently in the simple task described here, humans estimate risk and reward quite poorly under some circumstances. A better understanding of the neural mechanisms underlying efficient estimation of reward may yield insights into why these mechanisms sometimes fail. In applying decision models to combined neurophysiological and behavioural experiments, Platt and Glimcher have begun to develop an important new framework for studying these issues in future experiments. □

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Physiology

Crossing over to the dark side

Some locust species have a Jekyll-and-Hyde streak. Normally, they are a cryptic shade of green — shown in the photograph of *Schistocerca gregaria*, top — and lead solitary, unobtrusive lives. But at high population densities, their colour darkens and they become the gregarious, mobile animals that lay waste to crops. Now Amer Tawfik *et al.* have identified the hormone that triggers one aspect of this transmutation (Proc. Natl Acad. Sci. USA 96, 7083–7087; 1999).

Tawfik *et al.* used an albino mutant of the plague locust *Locusta migratoria* (middle), which did not darken when crowded. They found that implanting either the brain or the corpus cardiacum — an organ close to the brain — from normal locusts into the albino caused the production of melanin in the insect's cuticle, and the normal darkening response. The hormone is probably made in the brain, then transferred to the corpus cardiacum for storage and release.

Next, Tawfik *et al.* used the albinos to identify the messenger itself. They injected albino locust nymphs with purified extracts from normal locusts: any that darkened had received the active ingredient. It turns out that this is a short peptide, 11 amino acids long, called [His⁷] corazonin. The bottom photograph shows an albino nymph of *L. migratoria* after



injection with corazonin (the effect on the green *S. gregaria* is similar). This hormone had already been found in another locust species, but its function was not known.

The hormone, christened 'dark-colour-inducing neurohormone', produces its effects even in locusts that are not crowded — the group is going on to investigate its effects on behaviour. Intriguingly, its amino-acid sequence is similar to that of the melanophore-stimulating hormone (MSH) found in mammals, and injecting MSH into albino locusts also causes their colour to darken.

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Human evolution

We are what we ate

Bernard Wood and Alison Brooks

Much of evolutionary history involves the fine-tuning of variations on a common theme. But every now and again significant innovations (known as grade shifts) occur, forming the basis for the next phase of evolutionary activity. Evidence of such a grade shift in human evolution can be found in the fossil record of a little less than two million years (Myr) ago. Here we see the first signs of hominins (human ancestors from the tribe Hominini) whose bodies, jaws and teeth begin to resemble those of modern humans. Writing in the *Journal of Human Evolution*, O'Connell and colleagues¹ now challenge conventional wisdom by suggesting that the cascade of changes — involving anatomy, life history, culture and social structure — that resulted in the evolution of modern humans was set off not by hunting, but by systematic exploitation of subterranean food sources and recruitment of grandmothers to provide for their grandchildren.

Only the rash would claim that we understand the details of human evolution. Nonetheless, we can begin to sketch out a hypothetical evolutionary history (Fig. 1) that is unlikely to be badly wrong. For example, we're now almost certain that modern humans and chimpanzees diverged from a common ancestor² — who was chimp-like, forest-dwelling, and predominantly arbore-

al and fruit-eating — between 5 and 8 Myr ago. Although we'd expect human fossils to be considerably more bipedal than (and, thus, readily distinguishable from) the ancestors of chimps, this may not be so. Instead we may have to rely on the size and shape of the canines, as well as relatively subtle indicators in the deciduous and permanent post-canine teeth, to sort the first humans from the earliest chimps.

The most primitive hominin known, the 4.5-Myr-old *Ardipithecus ramidus*³, has relatively few derived hominin features. It is probably just one of a number of early hominins with different combinations and degrees of emphasis on 'human' features such as thickened enamel, upright posture and skeletal adaptations to a more terrestrial locomotion. After *A. ramidus*, but before 2 Myr ago, all species of hominin are megadont (that is, they have larger chewing teeth) compared with living modern humans and chimpanzees. But they differ in absolute and relative brain size, in the degree of megadonty, and in the extent to which the post-cranial skeleton shows adaptations to an upright posture and bipedalism⁴. There are probably undiscovered hominin species with different combinations of these adaptations.

There is relatively little hominin fossil evidence from between 3 and 2 Myr ago. However, this sparse evidence includes at

least three species in East Africa alone^{5,6}, and it is likely that more will be discovered. The post-cranial evidence from this, and earlier, periods suggests that the hominin species concerned were not committed bipeds⁷, as all retained some arboreal capability. Even though two of this group of hominin species, *Homo habilis* and *H. rudolfensis*, have been included in our own genus, they are as close — if not closer — to the australopithecines (bipedal primates of the genus *Australopithecus*). There are no sound reasons for continuing to include these taxa in *Homo*⁸.

A little less than 2 Myr ago we see tentative evidence in the fossil record of a new kind of hominin, known as 'early African *H. erectus*' or *H. ergaster*⁹. This is closer to later *Homo* taxa than it is to the australopithecines. It is the first hominin with a large body, fewer differences between the sexes, and a post-cranial skeleton that suggests it is fully upright and a committed biped. Relative to its body mass, its jaws and teeth are no larger than those of modern humans and chimpanzees. Moreover, its teeth, like those of modern humans, grow relatively slowly. It is only in the relatively small size of its brain that *H. ergaster* fails to conform to the pattern of later *Homo*⁸.

This huge shift in hominin morphology cannot be linked to the introduction of stone-tool manufacture or hunting — simple stone artefacts have been recovered from 2.5-Myr-old sediments¹⁰, and sound evidence for organized hominin hunting (as opposed to opportunistic scavenging) appears much later, in the Middle Pleistocene (780,000–130,000 Myr ago). Instead, O'Connell *et al.*¹ now suggest that, soon after 2 Myr ago, the cooler, drier and more seasonal climate would have made fruit a less dependable source of food. These conditions would favour any hominin that could efficiently harvest the underground storage organs, mainly tubers, that are relatively abundant in savannah habitats. That is, the larger-bodied, taller *H. ergaster* would have been better adapted for this task than its australopithecine ancestors and cousins.

O'Connell and colleagues' suggestion stems from observations made during their study of the Hadza, who live around Lake Eyasi in northern Tanzania. The Hadza are one of the few remaining populations practising traditional foraging. The authors noted the importance of post-menopausal grandmothers, who contribute to their own fitness by using foraged tubers to support both themselves and the weaned children of their daughters. In this way, the daughters are released to become pregnant again¹¹. O'Connell *et al.* argue that, during evolution, this practice would have favoured a longer lifespan, which, in turn, triggered a cascade of changes including the increased body mass, extended growth period and delayed maturity¹² seen in *H. ergaster*.

Complex behavioural hypotheses are

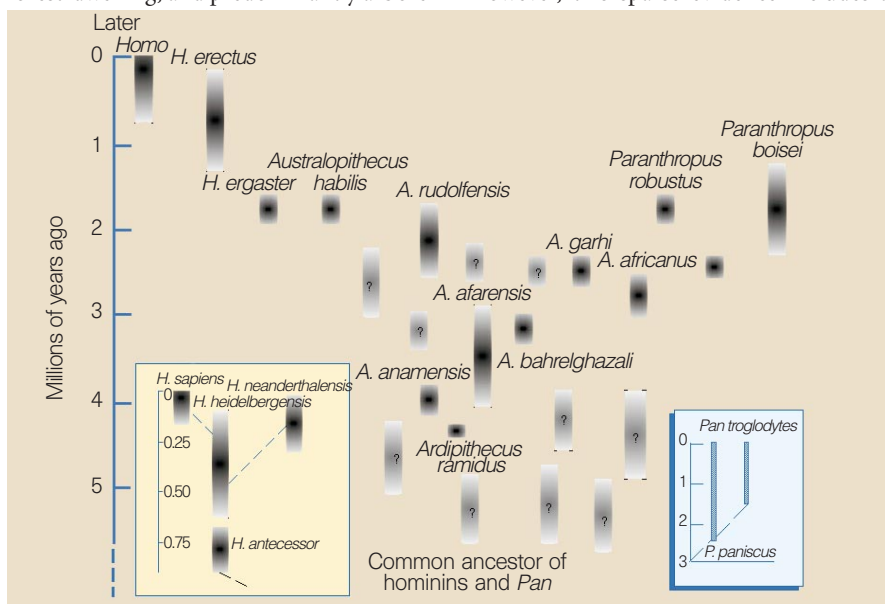


Figure 1 Species considered to be part of the tribe Hominini, or hominins, as opposed to chimpanzee ancestors, or panins. The horizontal axis spreads the species out according to the relative size of their chewing teeth and brain size — taxa with large molar and premolar crowns are to the right, and those with smaller post-canine teeth are to the left. O'Connell and colleagues¹ suggest that the grade shift between *Australopithecus* and *Homo*⁸ was initiated by the systematic exploitation of subterranean food sources, rather than by hunting, as was traditionally thought. The hypothetical taxa (?) are a reminder that the number of taxa will probably increase.

notoriously difficult to test. O'Connell and colleagues' proposal may, or may not, fit with the fossil and archaeological evidence, but possible tests include stable-isotope analyses to check for any dietary shift, and a search for residues of woodworking or starch grains on appropriate stone artefacts. Although the contemporary stone tools would not be obviously effective for hunting, they would have been adequate for preparing wooden digging sticks. *Homo ergaster's* body shape indicates that it is the first hominin to disperse with a large digestive tract, so its diet must have differed from that of its australopithecine predecessors, who, apparently, had a large gut like the living apes¹³. Moreover, *H. ergaster's* relatively small jaws and teeth are consistent with a diet requiring lower bite forces and less chewing. Wood and Collard¹⁴ and Wrangham *et al.*¹⁵ suggest that these changes are related to processing of food outside the mouth, through cooking. This proposal is also put forward by O'Connell *et al.*¹, who review the archaeological evidence for hominin-controlled fires.

The attraction of the foraging hypothesis is that it is the first attempt to link the nexus of morphological, life-history and cultural

changes that we see in the hominin fossil record a little less than 2 Myr ago. By playing down the role of meat eating at this stage in hominin evolution, O'Connell and colleagues' hypothesis raises the possibility that the increased body mass owing to foraging, combined with access to a more reliable source of meat, allowed the increase in absolute and relative brain size that was the next major innovation in human evolution.

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Although most of the activity associated with galaxy formation took place in the distant past, there are good reasons for believing that the outer reaches of galaxies are still in formation, at least in backwaters like the Local Group⁶. Many astronomers are tantalized by this possibility, not just because it may confirm our basic understanding of galaxy formation, but because of the chance to identify truly 'primordial' structures in the near field.

So where are these primordial building blocks? Blitz *et al.*² claim to have the answer. More than one-third of the sky is peppered with compact clumps of neutral hydrogen whose motions depart fairly radically from Galactic rotation. Discovered in 1963, these 'high-velocity clouds' (HVCs) have been the subject of wide-ranging speculation ever since. In the 1970s it became clear that a large fraction of HVCs fall along the Magellanic Stream, a thin band of gas encircling the Milky Way, which was presumably stripped from the Magellanic satellite galaxies⁷. After excluding these clouds, Blitz *et al.* argue that the motions of the remaining HVCs reflect the gravity field of the Local Group rather than our Galaxy. This puts their average distance at hundreds of kiloparsecs (1 kiloparsec = 3,262 light years), or far beyond the Galaxy. As a consequence, these clouds are typically 20 kiloparsecs and roughly 30 million solar masses of hydrogen in size, with potentially ten times more mass in the form of dark matter.

The Blitz *et al.* picture is very attractive because it unites ideas and observations that have been shelved for years. As early as 1966 Jan Oort⁸ noticed that the so-called virial

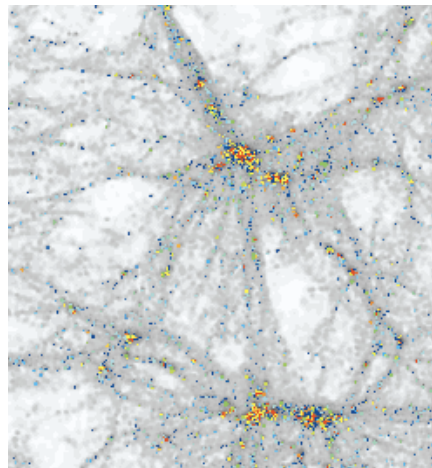


Figure 1 How the Universe might appear today, from a state-of-the-art *N*-body simulation by the Virgo Consortium¹². This thin slice is millions of light years across. The 'cold dark matter' clumps are shown in grey; red objects are galaxies consisting mostly of old stars; blue galaxies are still forming new stars at the present time. Blitz *et al.*² argue that clouds of gas and dark matter are galactic building blocks falling into the Local Group of galaxies to which our own Milky Way belongs.

Cosmology

Clues to galaxy formation

J. Bland-Hawthorn

In 1977, Stephen Weinberg observed that "the theory of the formation of galaxies is one of the great outstanding problems of astrophysics, a problem that today seems far from solution"¹. Although the past two decades have seen considerable progress, many questions remain. Broadly speaking, the quest for answers has followed two paths: near-field cosmology (looking for clues close to home) and far-field cosmology (looking back in time (redshift) for the progenitors of modern-day galaxies). In their latest joint venture, Leo Blitz, David Spergel and their colleagues² propose that an important clue may have been in plain view — that is, in the near field — for almost 40 years.

We know through direct observation that the Universe was vastly hotter and denser in the distant past than it is today. As the Universe expanded it cooled to a point where atomic hydrogen distilled out of the primordial plasma. A vast literature of theoretical work, aided by supercomputer simulations, has concentrated on what happened next. Here we must acknowledge the primary role of dark matter in driving galaxy formation, as it accounts for more than 90% of the mass in the Universe. Although the nature of dark matter is a complete mystery, the consequences for galaxy formation are radically different depending on whether dark matter is 'hot' or 'cold' (or a mixture of both). We

now know that dark matter must be mostly cold in order to produce the small-scale structure we see today in three-dimensional galaxy distributions³.

The modern paradigm is that when the Universe was cool enough to form atoms, much of the dark matter existed in small clumps. As time progressed, gravity caused these clumps to cluster together to form bigger systems, and onwards to galaxies. Supercomputer simulations have become an essential tool for understanding how cosmic evolution progresses in a hierarchical universe⁴.

When looking at such simulations (Fig. 1) it is important to keep in mind our humble vantage point. We live on the outer reaches of a very ordinary spiral galaxy within the Local Group, a motley collection of 40 or more (mostly small) galaxies. Our Galaxy and the Andromeda Galaxy dominate this group, accounting for more than 80% of the starlight. The Local Group is but a small subset of a much larger complex of galaxies known as the Coma-Sculptor Cloud, which in turn forms a small part of the Local Supercluster⁵. Supercluster scales are the largest entities modelled in computer simulations, and extend over distances of several hundred million light years. In short, we find ourselves in a sparse environment, somewhere along one of the connecting bridges that join the dense clusters.

theorem, which relates the gravitational energy of an object to its kinetic energy, would place many HVCs outside the Local Group. For each cloud, radio observations obtain a spectrum of the hydrogen emission line at a wavelength of 21 cm. The total intensity of the line is directly related to the cloud's mass and distance. For a self-gravitating cloud, the intrinsic width of the spectral line is also related to the cloud's mass but has a different dependence on distance. Presumably the inferred masses are the same, in which case a crude 'virial' distance can be determined. This distance depends on a basic assumption that the clouds are held together by gravity, and therefore an independent distance estimate is called for.

One method is to look for bright sources with well-calibrated distances along the line of sight to a gas cloud. If the cloud produces absorption in the visible spectrum of the more distant source, but not in the spectrum of the nearer source, the distance to the HVC can be bracketed⁹. At present, this method has been applied only to background sources within the Galactic halo.

Another method has the potential to reach much greater distances. A spate of new surveys (for example, see ref. 10) reveals that HVCs along the Magellanic Stream are easily observed from visible emission lines of hydrogen. Whereas the radio spectrum is produced by neutral atoms, the visible spectrum arises from hydrogen ionized after absorbing ultraviolet photons. The latest observations (M. E. Putman and J. B.-H., unpublished work) show clearly that the clouds are statically photoionized, presumably by young stars in the Galaxy¹¹. Clouds at even greater distances than the Magellanic Stream — that is, beyond 50 kiloparsecs — should have even weaker ionized hydrogen emission. Much beyond 300 kiloparsecs, it would be very difficult to detect any ionized hydrogen signal at all.

The discovery of dense HVCs with no detectable signal for ionized hydrogen would constitute the first tentative step in demonstrating that there may indeed be a population of primordial gas clouds (with associated dark matter) dispersed throughout the Universe, thereby confirming Blitz and colleagues' hypothesis. These are exciting times for near-field studies of galaxy formation. Several groups around the world are well placed to identify a statistically useful sample of such clouds for closer scrutiny with the new generation of large ground-based and space-based telescopes. Watch this space. □

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Biophysics

Relating dynamics to function

Ann Stock

Movement at the molecular level is essential for macromolecular function. Nonetheless, our images of proteins are dominated by static, three-dimensional structures that give only snapshots of the allowable conformations. One method for studying the structural dynamics of macromolecules in solution is NMR spectroscopy, which is providing increasingly detailed descriptions of the motional parameters in proteins¹. But it is not straightforward to correlate molecular motions with protein activities, and a central question remains — how do dynamics relate to function?

On page 289 of this issue, Feher and Cavanagh² give an insight into this problem with their NMR dynamics study, which characterizes movements in Spo0F, a response-regulator protein involved in a phospho-relay signal-transduction pathway in *Bacillus subtilis*. Feher and Cavanagh find that amino-acid residues moving on microsecond to millisecond timescales in Spo0F correspond to residues that have previously been identified as important in mediating interactions with three other signalling partners^{3,4}. This study adds to the growing list of cases where conformational dynamics are correlated with protein–protein interaction surfaces.

The subject of the study, Spo0F, belongs to a large family of response-regulator proteins that function within two-component signal-transduction pathways⁵. These two-component systems involve a sensor kinase that adds a phosphoryl group to itself (autophosphorylates) at a histidine residue. This creates a high-energy phosphoryl group that is then transferred to a conserved regulatory domain of a response-regulator protein. Two-component systems are modular with respect to both the arrangement of domains within proteins, and to proteins within pathways. But whereas many such systems involve just a sensor kinase and a response regulator, others, such as the *B. subtilis* sporulation pathway in which Spo0F participates⁶, use many phospho-transfer components in a more complex phospho-relay scheme.

The conserved response-regulator domain acts as a phosphorylation-activated switch, in which the phosphorylated form typically corresponds to the active, or 'on', state of the protein. Diverse experimental

data obtained with different response regulators has led to the generally accepted hypothesis that the regulatory domain can exist in two main conformations, with phosphorylation shifting the equilibrium between them. Regulatory domains then modulate the activity of other, 'effector' domains, owing to different protein–protein interactions favoured by the two switch conformations. Unfortunately, the short lifetime of phosphorylated response regulators (typically ranging from seconds to hours) has so far inhibited their direct structural analysis.

Spo0F is a small, 124-amino-acid protein consisting of five α -helices and five β -strands joined by small loop regions. It interacts with three different proteins in the sporulation pathway — its upstream and downstream phospho-relay partners (histidine kinase, KinA, and phosphotransfer protein, Spo0B, respectively), as well as with a phosphatase, RapB. Residues involved in these interactions have been identified by alanine-scanning mutagenesis, and they form a semi-contiguous surface that extends over one face of Spo0F^{3,4}. When Feher and Cavanagh compared this functionally defined surface to their dynamics data, they found a strong correlation.

The authors determined NMR relaxation parameters for most of the backbone amide nitrogens of Spo0F, then they fitted these to dynamic models⁷. They obtained estimates for the magnitude and timescale of backbone motions. The parameters indicate that the α -helices and β -strands in Spo0F (shown, in Fig. 1a and b, as helical ribbons and arrows, respectively) behave rigidly in the picosecond to nanosecond timescale. This contrasts with regions at the amino and carboxy termini, and a loop extending from the phosphorylation site, which undergo fast internal fluctuations. The authors found that regions which experience perturbations in the microsecond to millisecond range form a distinct patch that almost perfectly overlaps the previously defined protein–protein interaction surface (Fig. 1).

How do these micro- to millisecond fluctuations contribute to Spo0F function? This question provides Feher and Cavanagh with a fertile topic for speculation. They address the possibility that the flexibility of regions involved in protein–protein interactions

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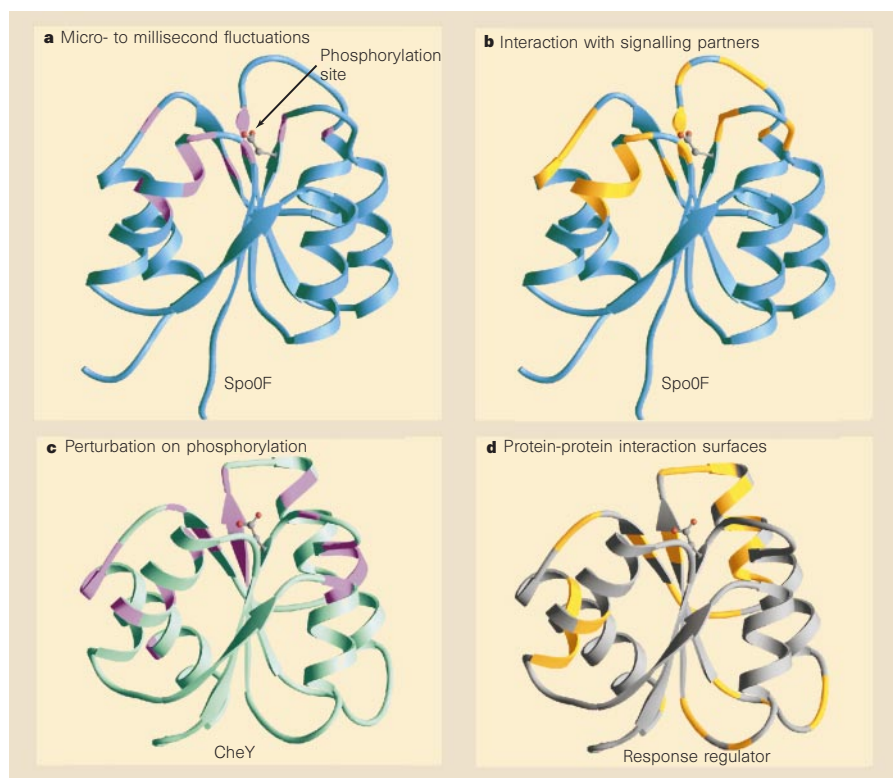


Figure 1 Dynamic regions in response-regulator proteins. **a**, Spo0F, a signalling protein that participates in the phospho-relay pathway controlling sporulation in *Bacillus subtilis*. Magenta regions indicate residues that Feher and Cavanagh² find have motions in the microsecond to millisecond timescale. **b**, These residues are compared to a surface of Spo0F that has previously been identified as important for interactions with other signalling partners^{3,4} (gold). **c**, Residues in the response regulator CheY that show large chemical-shift changes on phosphorylation⁸ (magenta). **d**, Residues involved in protein-protein interactions in the response regulators CheY, CheB, NarL and PhoB (gold) mapped onto a representative domain as described previously¹².

may facilitate binding of different partners. They also suggest that different entropic contributions in the bound and free states may contribute to modulation of binding affinities. (Advances in analysing dynamics at macromolecular interfaces should give a more detailed assessment of these entropic contributions.) Alternatively, the mobility in Spo0F may be related to the conformational changes associated with phosphorylation. The structural fluctuations could reflect the many conformational substates of Spo0F, one of which probably corresponds to the active conformation.

It may not be feasible to distinguish the relative contributions of dynamics to each of these functional features of Spo0F, because phosphorylation-induced conformational changes and protein-protein interactions are intertwined in the family of response-regulator proteins. Take, for example, NMR chemical-shift-perturbation studies of two other response regulators, CheY and NtrC, which indicate that phosphorylation induces a propagated conformational change that extends over one face of the domain^{8,9}. Protein-protein interaction surfaces identified in several different response regulators overlap with this region (Fig. 1). Perhaps unsurprisingly this surface corresponds with the

flexible regions in Spo0F, and the fundamental strategies for its functioning will probably turn out to be common to all response-regulator proteins. In the meantime, the forthcoming structural characterization of Spo0F complexed with its partners¹⁰, as well as the structural and dynamic characterization of a phosphorylated response regulator¹¹, should increase our understanding of this functionally and structurally dynamic surface in response-regulator proteins. □

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Daedalus

Reason to be cheerful

The ultimate goal of civilization is, perhaps, maximizing the sum of human happiness. Most governments interpret this as raising the standard of living. In fact, happiness correlates fairly weakly with economic affluence. For most of us, it is pretty much set by our inherent temperament, sunny or gloomy, modified by the triumphs or disasters of the past six months or so. Even lottery winners and bankrupts relax back to their normal mental state in under a year.

So Daedalus wants to lift our spirits directly. 'Cosmetic psychopharmacology' — the use of anti-depressant or narcotic drugs — has serious social side-effects. But Daedalus recalls the story of a patient with a fairly non-invasive pancreatic tumour. Despite his unenviable plight, the man was permanently cheerful. It turned out that the tumour generated a steady stream of endorphins, the hormones that mediate our feelings of satisfaction. This suggests that our inherent personality differences merely reflect the different endorphin levels maintained by our bodies.

Few people would want to boost their happiness by means of an implanted tumour, however non-invasive. Gene therapy seems more promising. Some of the subject's own cells would be extracted, loaded with the genes for his own endorphins, and then re-implanted into him. Dermal cells would be a good choice. They are easy to get at, and can be reimplanted by simple tattoo technology. If the subject remained gloomy, more cells could be inserted; if he developed a dangerously manic optimism, some could be removed.

Gene therapy, of course, is in its infancy. It is tricky to insert the right genes into the chosen cells, and trickier still to switch them on. But resources put into developing Daedalus' scheme should bring far greater returns of happiness than any amount of economic growth. Governments should rush to fund the project and offer its benisons to the people, starting with the most disgruntled and resentful citizens: criminals and the underclass. Infused with calm self-satisfaction, they would cease to be so troublesome. Later, the long-term unemployed and the more clamorous political activists would be added; later still, the rest of us. As contentment spread through society, the drive and restless ambition that fuel, not only crime, but innovation and economic growth, would falter and fade. But that, of course, would no longer matter.

David Jones

Too good to be true

Many a scientist has been seduced by an elegant idea, only to find that aesthetics are not always a good guide to a theory's accuracy. Of all the beautiful theories slain by ugly facts, which most deserved to be right?

John Maynard Smith

We have all had the experience of having what seemed like a great idea, only to find that it wouldn't do, because it was contradicted by a familiar fact or suffered from an incurable logical error. The experience prompts the question: what was the cleverest idea in the history of science that turned out to be wrong?

Before you try to answer this question, let me insist that by 'wrong' I mean really wrong. I won't accept 'Newton's laws of motion' as an answer. I understand that physicists nowadays insist that his laws break down at very high velocities and accelerations. As an ex-engineer whose aeroplanes never flew faster than sound, let alone light, I have great faith in Newton's laws: they are not so much 'wrong' as true subject to certain constraints. For the same reason, I would not accept 'Mendel's laws' as an answer either, even though the law of independent assortment is false for genes on the same chromosome. I want an idea that is wrong in all circumstances, but which deserves to be right.

I would not be asking this question if I did not have a candidate answer. In the physical sciences, one could suggest Kelvin's estimate of the age of the Earth, based on the expected rate of cooling, but wrong because Kelvin knew nothing of radioactive heating. The idea was wrong, and important, but perhaps not terribly clever. A better candidate, I think, would be the steady-state theory of the Universe. I find it a much more attractive notion than the Big Bang, but I have to take it on trust that it is mistaken.

My favoured candidate is from biology. In 1957, Francis Crick, John Griffith and Leslie Orgel published a paper explaining why only 20 amino acids are used in proteins, although other possible amino acids exist (*Proc. Natl Acad. Sci. USA* **43**, 416–421; 1957). It was already known that the sequence of amino acids in a protein was determined by the sequence of four kinds of base — A, C, G and T — in DNA, but the 'code' connecting the base sequence to the amino-acid sequence was unknown. It was widely assumed that this would be a triplet code. It could not be a doublet code, as at best this could specify only 16 amino acids. The idea that it resembled Morse code, with different numbers of bases specifying different amino acids, although possible, was not thought likely.

The authors argued as follows. Granted that it is a triplet code, how does the cell know which triplets to read? There are no 'spaces' in DNA to separate them, analogous to the



No matter in what order these meaningful triplets are arranged, none of the 'out of frame' triplets must be meaningful.

spaces that separate letters in Morse code. It is implausible to suppose that translation starts at the beginning of a gene and counts off in threes. Instead, they suggested that only some of the triplets are meaningful, in the sense of specifying an amino acid. No matter in what order these meaningful triplets are arranged, none of the 'out of frame' triplets must be meaningful. For example, if ACT and GCA are meaningful, then CTA, TAC, AGC, CAG, CTG, TGC, CAA and AAC must not be. Then a message can be read in only one way. If that is how it works, what is the largest number of meaningful triplets that is possible? The answer, of course, is 20.

The authors treated their idea with due caution, writing: "We present the solution here because it gives the 'magic number' 20, so that our answer may perhaps be of biological

significance". If it had been my idea, I would be believing it still. But we now know that 61 of the 64 triplets specify an amino acid. The cell does indeed start at the beginning of a gene and count off in threes. If one or two nucleotides are deleted, then every amino acid beyond the deletion is wrong, and the protein is useless. If three are deleted, translation gets back into frame, and all is not lost.

It is a remarkable twist in the history of science that Crick (pictured) was one of the authors of the paper that used this argument to show that the code is indeed a triplet code, and that the cell does count in threes (Crick, F. H. C., Barnett, L., Brenner, S. & Watts-Tobin, R. J. *Nature* **192**, 1227–1232; 1961).

Since writing the above, I find I am not alone in my choice. Twenty years ago (what is it about 20?), describing the above argument, Horace Freeland Judson wrote: "This was the beginning of the comma-free code — an idea of Crick's that was the most elegant biological theory ever to be proposed and proved wrong" (*The Eighth Day of Creation*, Cape, 1979).

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Prions prevent neuronal cell-line death

Prion diseases, such as scrapie and bovine spongiform encephalopathy (BSE) in animals and Creutzfeldt-Jakob disease (CJD) in humans, are neurodegenerative conditions characterized by the accumulation of a post-transcriptionally modified, pathological form of a host-encoded glycoprotein, designated PrP^{Sc}. The physiological function of the normal cellular isoform, PrP^C, is unknown, although studies of mice devoid of PrP^C have indicated that it may be involved in normal synaptic function and survival of Purkinje cells, but findings have been inconsistent¹⁻⁶. We find that serum removal from the cell culture causes apoptosis in *Prnp*^{-/-} cells (in which a disrupted form of the prion protein is produced) but not in *Prnp*^{+/+} (wild-type) cells. Transduction of PrP or the Bcl-2 gene suppressed apoptosis of *Prnp*^{-/-} cells under serum-free conditions. We also found that *Prnp*^{-/-} cells extended shorter neurites than *Prnp*^{+/+} cells, but expression of PrP^C increased their length. These findings support the idea that the loss of function of PrP^C may partly underlie the pathogenesis of prion diseases.

We established hippocampal cell lines from *Prnp*^{-/-} and *Prnp*^{+/+} mice. In the mutated allele, about 800 base pairs of intron 2 and the entire open reading frame (ORF) of *Prnp* were replaced with a *pgk-eo* gene cassette. Primary cultures of hippocampal cells from 14-day-old *Prnp*^{-/-} mouse embryos were used as a target for infection with a retrovirus vector containing

a gene encoding SV40 large T antigen⁷. We studied three *Prnp*^{-/-} cell lines (HpL2-1, HpL3-2 and HpL3-4) and three *Prnp*^{+/+} cell lines (HW8, HW9 and HW18) from several independent immortalized cell lines. The absence of PrP on the cell surface of *Prnp*^{-/-} cell lines was confirmed by negative immunoreactivity with a rabbit anti-PrP polyclonal antibody⁸.

Transcripts of the gene encoding a neurofilament of relative molecular mass 68,000 (NF-68K) were detected by the polymerase chain reaction with reverse transcription (RT-PCR) in RNA samples of all *Prnp*^{+/+} and *Prnp*^{-/-} cell lines treated with or without the phorbol ester TPA (also known as PMA). After treatment with TPA, transcripts of the NF-200K gene were also detected in three *Prnp*^{+/+} cell lines but in only one *Prnp*^{-/-} cell line (HpL2-1). The transcription of glial fibrillary acidic protein, a glial cell marker, was not detected in *Prnp*^{-/-} or *Prnp*^{+/+} cells. These results indicate that all six cell lines used belong to the neuronal-precursor cell lineage, although they are variable in their developmental stages.

In serum-free medium, neurite-like structures were extended within an hour in all cell lines (Fig. 1a, c). Within 4 days, all *Prnp*^{-/-} cells died (Fig. 1d), beginning with cells grouping together, followed by condensation of cytoplasm and retraction of neurites. In contrast, all *Prnp*^{+/+} cell lines maintained structural integrity under these culture conditions (Fig. 1b).

DNA fragmentation characteristic of apoptosis was seen in *Prnp*^{-/-} cells cultured in serum-free media, regardless of whether they were treated with TPA or dibutyryl cyclic AMP. DNA fragmentation was observed in the three *Prnp*^{-/-} cell lines, but not in the three *Prnp*^{+/+} cell lines, by agarose-gel electrophoresis (Fig. 1e, f). The DNA was extracted from cells cultured without serum, electrophoresed on 2% agarose gel, and stained with ethidium bromide. DNA ladders were first detected in *Prnp*^{-/-} cells cultured for 12 hours without serum.

To confirm the close relationship between PrP^C and apoptosis, we transfected a PrP^C expression vector (pMAM2-MPR-BSD) into the *Prnp*^{-/-} cell lines (HpL2-1 and HpL3-4). The vector carried the ORF of *Prnp* under the control of Rous sarcoma virus and mammary tumour virus long terminal repeats and the blasticidin-S deaminase genes with an SV40 promoter. The expression vector does not contain any intron sequences from *Prnp* gene. The successful transformants were cultured for 10 days with 8 µg ml⁻¹ blasticidin-S. The selected cell lines, designated as HpL2-1TR and HpL3-4TR, survived under serum-free conditions without any sign of apoptosis (Fig. 1g). Immunofluorescence studies showed positive staining of PrP^C in HpL2-1TR and HpL3-4TR cells, but not in their non-transfected counterparts. Transfection with pMAM2-BSD lacking the *Prnp* ORF did not suppress apoptosis in HpL2-1 or HpL3-4 cells. These results indicate that the apoptosis of *Prnp*^{-/-} cells was prevented not only by factors in the serum, but also by the expression of PrP^C.

The caspases (a family of cysteine proteases) are the principal executors of apoptotic cell death, but many molecules are known to modulate the caspase cascade. One of these, Bcl-2, has been reported⁹ to interact with PrP^C in the two-hybrid system with yeast¹⁰. Although there is no evidence for direct binding of PrP^C to Bcl-2 in vertebrate cells, our preliminary experiments indicate functional interactions between PrP^C and Bcl-2. *Prnp*^{-/-} cells transfected with pMAM2-Bcl2-BSD and overexpressing murine Bcl-2 survived without any sign of apoptosis in selected cell lines designated as HpL3-4TRBcl-2 (Fig. 1h).

As well as undergoing cell death, our *Prnp*^{-/-} cell lines show significantly shorter neurite extension than *Prnp*^{+/+} cell lines after treatment with TPA. Again, after transfection with pMAM2-MPR-BSD, the *Prnp*^{-/-} cells recovered the ability to extend neurite-like structures (data not shown).

Our results support the idea that the loss of PrP^C function plays some pathogenic role

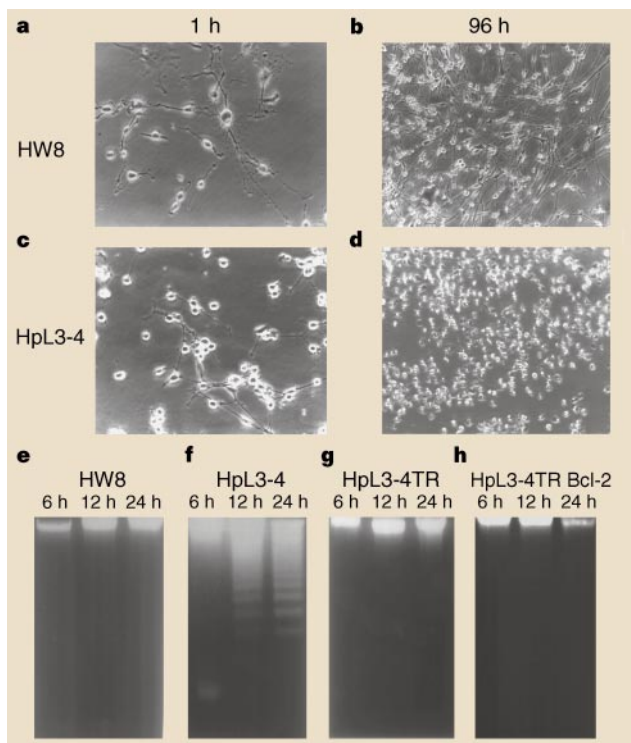


Figure 1 Morphological appearance of HW8 *Prnp*^{+/+} and HpL3-4 *Prnp*^{-/-} cells. **a, b**, HW8 cells were cultivated in 1 mM dcAMP-containing medium for 1 h (**a**) or 96 h (**b**), when viable differentiated neuronal-like cells were observed. **c**, HpL3-4 cells cultured for 1 hour showed shorter neurite-like extensions. **d**, HpL3-4 cells, after 96 hours, show cell death, with round, condensed and irregularly shaped cell bodies. **e-h**, PrP^C-dependent apoptosis under serum-free condition revealed by DNA fragmentation. **e**, HW8(*Prnp*^{+/+}); **f**, HpL3-4(*Prnp*^{-/-}); **g**, HpL3-4TR; **h**, HpL3-4TRBcl2. Cells were cultured for the time shown in serum-free conditions. DNA fragmentation was observed in HpL3-4 cells only.

in prion diseases. Because the serum-free supernatant of *Prnp*^{+/+} cell cultures could not rescue the phenotypes of *Prnp*^{-/-} cells, PrP^C might act autonomously. Our *in vitro* system provides a new way to detect the cellular function of PrP^C in nerve cells and to establish the molecular mechanisms of apoptosis involving PrP^C.

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Breast-cancer diagnosis using hair

James *et al.*¹ observed a difference between the X-ray diffraction patterns of hair from healthy females and from breast-cancer patients. This difference was reported as the presence of an extra ring corresponding to a spacing of 4.44 nm on the patterns obtained from breast-cancer patients. This ring was also observed in patterns from subjects “not yet diagnosed with breast cancer but suspected of being at risk”. James *et al.* proposed that these observations might lead to a screening method for breast cancer. We have now repeated the study using a different hair type (from the scalp), but are unable to replicate their observations.

The existence of a ring at a spacing of 4.5 nm was first attributed to ‘lipid crystals’² that could be removed only by prolonged extraction in hot solvents. Our experience^{3,4} and that of our colleagues from l’Oréal⁵ confirms the presence of intense diffraction ring(s) arising from lipids in keratinous tissues. We recently performed a microdiffraction analysis on beamline ID13 at the ESRF

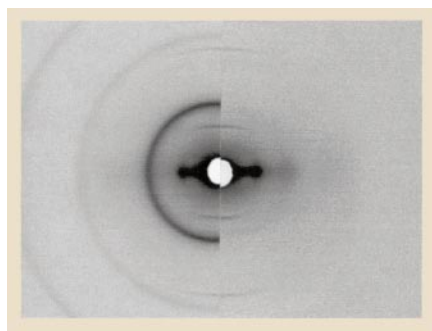


Figure 1 Split X-ray diffraction pattern. Left, scalp hair from healthy subject, displaying intense rings arising from lipid granules; right, hair from breast-cancer patient, with no clear rings.

and observed in human hair and other tissues that the lipid granules are mainly located in the outer part of the tissue⁶. We are therefore unable to explain the lack of the ring at 4.5 nm in the diffraction pattern of normal hair reported by James *et al.* One possibility is that working with a microbeam inhibited the observation of the ring because the lipid granules took up unfavourable orientations.

To compare X-ray diffraction patterns of hair from healthy subjects and breast-cancer patients under the same experimental conditions, we conducted experiments at station D43 of the synchrotron source LURE (Université Paris-Sud). We compared diffraction patterns of scalp hair from ten supposedly healthy people (seven females and three males) with the patterns from ten breast-cancer patients (all female). We irradiated a bundle of hair in a glass capillary with a 0.5-mm monochromatic X-ray beam. The diffraction patterns from healthy subjects displayed an intense ring at 4.48 ± 0.05 nm. The variability of the patterns from breast-cancer patients was larger, but the ring was intense for one patient, of low intensity for seven others, and not observable for the remaining two.

Our observations differ from those of James *et al.* in two main respects. First, we observed the diffraction ring at 4.5 nm on patterns from all healthy subjects (100%, against 22% by James *et al.*). Second, the ring is present in eight out of ten patterns from breast-cancer patients (instead of the 100% observed by James *et al.*). This conclusion is illustrated in Fig. 1: in two out of ten cases, our split patterns between healthy and breast-cancer subjects are exactly the opposite of that of James *et al.* However, it should be remembered that the studies used different hair types.

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Editorial note Full experimental details of the work by James *et al.* have been published^{1,2}. Detailed set-ups for the three different beamlines and links are available from <http://www.ansto.gov.au/natfac/asrp.html>

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Evolution of cooperation between individuals

Nowak and Sigmund¹ conclude that cooperation may have evolved through indirect reciprocity by image scoring. Their simulations¹ and analytical models^{1,2} predict long-term cyclical dynamics between cooperative and defector populations rather than an evolutionarily stable equilibrium. Here we add a realistic feature to their model: that there are always some individuals unable to cooperate owing to their poor phenotypic condition (we call these individuals ‘phenotypic defectors’). The presence of phenotypic defectors paradoxically allows persistent discriminating cooperation under a much wider range of conditions than found by Nowak and Sigmund because there is selection against both defection and unconditional altruism. In real populations there will nearly always be some level of defection because phenotypic defectors (such as the young, sick or handicapped) may be unable to help even if they have a genetic predisposition to do so.

To test the effect of phenotypic defection, we analysed Nowak and Sigmund’s model¹ of indirect reciprocity. In their scheme, each individual has a genetic strategy (k) and a non-heritable image score (s). The image of an individual is crucial in determining whether or not it will receive help from others. The image score is zero at birth, increases by one with every cooperative interaction, and decreases by one with every defection (decision not to donate). In each generation, interactions between pairs of individuals occur at random, with donors cooperating only if the recipient’s image score is greater than or equal to the donor’s genetic strategy, k ($s \geq k$). The strategies of individuals are permitted to lie between $k = -5$ (unconditional cooperation) and

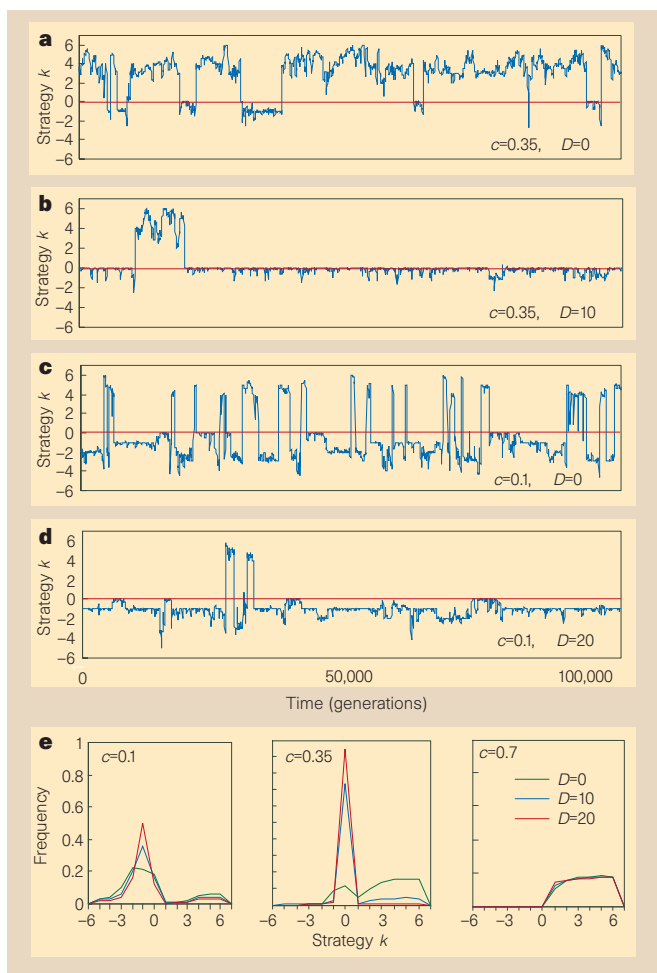


Figure 1 Computer simulations of the model¹ with $N=100$ individuals, D phenotypic defectors and different costs of cooperation c . **a–d**, Average strategy $\langle k \rangle$ of the population over a simulation of 100,000 generations for different c and D . **e**, Frequency distribution of strategies sampled over 10^7 generations with low, intermediate and high costs of cooperation, and with various numbers of phenotypic defectors, D . Model parameters are as in ref. 1, with $b=1$, mutation rate $p=0.001$, and $m=300$ random interactions per generation. As in ref. 1, after every generation the number of offspring of each player is determined by the fitness accumulated over a lifetime of interactions, and the population strategies evolve by selection and mutation over many generations.

$k = +6$ (pure defection), with $k=0$ indicating a ‘discriminating’ strategy. The cost to the donor’s fitness for each cooperation is c and the recipient’s benefit is b .

We modified this scheme and allowed a population of $N=100$ individuals to carry D phenotypic defectors. We assigned the non-heritable phenotypic strategy $k = +7$ to D randomly chosen individuals every generation, but we left unchanged the heritable genotypic strategies of these defectors.

We first consider the effects of phenotypic defectors in a model in which the costs of cooperation are intermediate compared with the benefit of the beneficiary ($c=0.35$, $b=1$). In the absence of phenotypic defectors ($D=0$), the simulation model¹, which incorporates mutation and drift, generally results in a population consisting almost entirely of defectors (Fig. 1a). However, upon the introduction of $D=10$ phenotypic defectors, the population structure becomes dominated by discriminating altruists whose average strategy is mostly $\langle k \rangle = 0$ (Fig. 1b). Over 10^7 generations, the introduction of phenotypic defectors increases the overall frequency of discriminators ($\langle k \rangle = 0$) from 12% ($D=0$) to 74% ($D=10$) and even 95% ($D=20$) (Fig. 1e, middle). These results demonstrate that phenotypic defectors, who are unable to

provide help, may nevertheless be central to the evolution of cooperation.

We turn now to the model in which the cost of cooperation is relatively low¹ ($c=0.1$). With no phenotypic defectors ($D=0$), the simulation model yields long-term cycling in the average strategy index, generally with cooperative populations ($\langle k \rangle \leq 0$), but with defectors invading occasionally (Fig. 1c). Although the population initially consisted mostly of cooperators, the introduction of 20 phenotypic defectors led to discriminating cooperators being favoured (at $\langle k \rangle = -1$), effectively suppressing this cycling dynamic (Fig. 1d).

The paradoxical effect whereby phenotypic defectors stabilize discriminate altruism can be explained analytically (see <http://lynx.tau.ac.il/coop.html>). Whenever there are defectors in the population, there is a persistent advantage for discriminators over non-discriminating altruists because discriminators avoid paying the costs of helping defectors. This advantage disappears as soon as all defectors are eliminated from the population, as often occurs in the Nowak and Sigmund model¹. In our model, however, a constant supply of phenotypic defectors ensures that the advantage persists, leading to a large population of discriminators that deny help to real genotypic defectors and hence block their invasion.

Our study also shows that the qualitative effect of phenotypic defectors is highly dependent on the cost of cooperation, c (Fig. 1e). A high cost ($c=0.7$) leads to a population of defectors ($\langle k \rangle > 0$) no matter how many phenotypic defectors D are added. However, for intermediate costs ($c=0.35$), the addition of only a few phenotypic defectors strongly increases discriminate altruism (Fig. 1b, e). For low costs ($c=0.1$), where the population is initially cooperative with long-term cycling (when $D=0$), the addition of phenotypic defectors tends to suppress the cycling dynamic.

In the Nowak and Sigmund model¹, the relative frequencies of discriminators and non-discriminators fluctuate during the long-term population cycling, whereas our model predicts that, in cooperative societies, non-discriminators will be replaced by discriminators when the cost of cooperation increases. This implies the evolution of a society in which cheap donations are given unconditionally to everyone, whereas more costly gifts are given discriminatingly, and only to those individuals who can afford to donate such gifts to others (those who are in good phenotypic condition). Evidence for costly help being provided unconditionally to poor phenotypes may not be explained by indirect reciprocity, but rather by alternative theories^{3–5}.

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Higher fullerenes in the Allende meteorite

Fullerenes (C_{60} and C_{70}) were discovered during investigations of the mechanism by which carbon molecules form in interstellar and circumstellar shells¹. Unlike diamond and graphite, the other pure forms of carbon, fullerenes are extractable in an organic solvent such as toluene, which led to the detection of the higher fullerenes (C_{100} to C_{250}) in carbon-arc-evaporated soot material². We have applied a similar solvent extraction procedure to an acid residue of the carbonaceous chondrite from the Allende meteorite to search for higher fullerenes. We found C_{60} and C_{70} , as well as a unique distribution of remarkably stable clusters of C_{100} to C_{400} . These large extra-terrestrial carbon clusters are either the first indication of higher fullerenes or are an

entirely new range of aromatic carbon-rich molecules.

Carbonaceous chondrites contain interstellar grains, including diamond, silica carbide (SiC), graphite and carbon-based molecules that were formed in stellar atmospheres, mixed into the Solar nebula, and incorporated into the meteorite matrix³. Fullerenes (C_{60} and C_{70}) measured in parts per billion, and possibly fulleranes ($C_{60}H_x$), have been detected in the Allende meteorite, indicating that they existed either in the early Solar nebula or as a component of pre-Solar dust^{4,5}. Polycyclic aromatic hydrocarbons (PAHs) may also form in circumstellar environments^{6,7}. Theories about the role of diamonds, graphite, SiC, PAHs, fullerenes and fulleranes have also been proposed to explain such phenomena as the diffuse interstellar bands.

Ascertaining how and where these carbon species form requires an assessment of the structure and stability of carbon clusters. For clusters of less than ten carbon atoms, linear structures are energetically favoured. At C_{10} to C_{20} , linear structures close into rings, but between C_{20} and C_{30} (the 'forbidden zone') it is unclear what structures are favoured⁷. For clusters with more than 30 carbon atoms, however, the lowest-energy structures, with up to several hundred carbon atoms, seem to favour aromatic and fullerene-related carbon cages^{7,8}. Because the size range of interstellar PAHs

is limited owing to hydrogenation (at about 30 carbon atoms, individual PAHs may be fully hydrogenated, slowing growth as carbon atoms are added), and only trace levels of C_{60} and C_{70} have been detected in a single carbonaceous chondrite^{4,5}, the search has now turned to the larger clusters (carbon 'onions' and nanotubes) as a possible carrier of noble gases and as a higher-molecular-mass carbon component in meteorites. We have searched for fullerenes and larger clusters in both the toluene extract and the acid-insoluble residue (extracted with 1,2,4-trichlorobenzene; TCB) generated from the Allende meteorite. Laser desorption (linear) time-of-flight mass spectrometry (LDMS) was used to detect fullerenes (C_{60} and C_{70}) and any higher-molecular-mass carbon clusters.

LDMS analyses of the toluene extract revealed peaks at C_{60}^+ and C_{70}^+ , confirming our earlier results⁵ on a separate acid-treated split of the Allende meteorite. In addition, C_{74}^+ , C_{76}^+ , C_{78}^+ and C_{84}^+ were detected at a factor of 2–3 below C_{60}^+ and C_{70}^+ , as well as several mass peaks in the m/z range 100 to 400 atomic mass units, indicating the presence of PAHs and their methyl derivatives^{4,5}. LDMS analysis (Fig. 1a) revealed peaks for C_{60}^+ , C_{70}^+ and some higher fullerenes (C_{76}^+ to C_{96}^+) and a more prominent high-mass envelope extending from C_{100}^+ to C_{250}^+ . Closer examination of the larger carbon clusters (Fig. 1b) revealed

a large repeating peak pattern separated by about 24 atomic mass units. These clusters are separated by C_2^+ units, indicating that the high-mass envelope detected in the toluene-insoluble TCB residue is due to pure carbon clusters².

This idea is supported by considering the composition of the TCB-extracted Allende residue. In general, the insoluble carbon component of carbonaceous chondrites (CI, CM, CO and CV) is a 'macromolecular material' containing hydrogen, nitrogen, oxygen, sulphur and perhaps halogens, as well as carbon⁹. However, the Allende meteorite appears to be the limiting case in that the principal insoluble component is elemental carbon, which differs from graphite in having a poorly ordered structure (sp^2 -hybridized carbon)¹⁰. It is this insoluble material that retains the high-mass carbon clusters seen in Fig. 1a,b when extracted with TCB. The Allende residue also has several well-ordered graphitic particles that are similar in size and appearance to carbon onions and nanotubes⁹.

It seems that fullerenes and higher-carbon clusters are indeed formed in the outflows of carbon stars, as was suggested soon after their discovery¹. Their presence in the Allende meteorite indicates that they can survive passage through space and subsequent processing in the interstellar medium, and the accretion process into grains and larger bolides (such as asteroids or meteorites), as well as arriving on planets such as Earth. Fullerenes and higher-carbon clusters may have been important on the early Earth, and perhaps other planets, by providing not only a source of carbon, an essential element for life, but also the volatiles that contributed to the planetary atmospheres needed for the origin of life¹¹.

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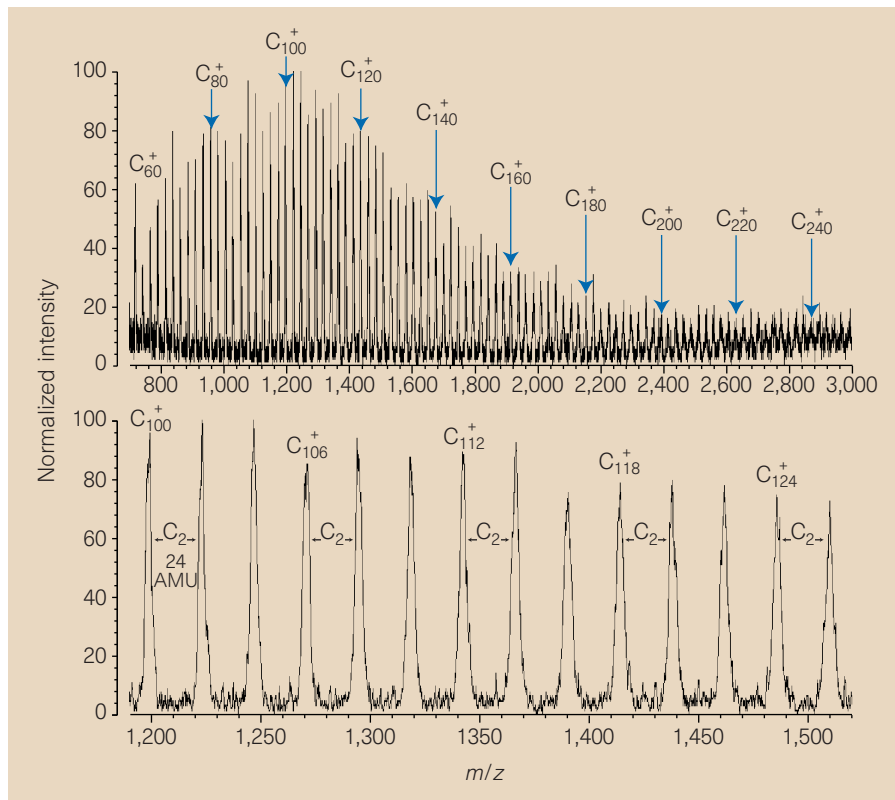


Figure 1 LDMS mass spectra for the TCB extract of the Allende meteorite. **a**, Spectra showing a small peak for C_{60}^+ and a range of remarkably stable larger carbon clusters between C_{100}^+ and C_{250}^+ (clusters were observed up to C_{300}^+). **b**, Closer examination of the larger clusters. For more details, see Supplementary Information.

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- Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

Learning from the rainbow

Colour and Meaning: Art, Science and Symbolism

by John Gage

Thames & Hudson: 1999. 320 pp. £32

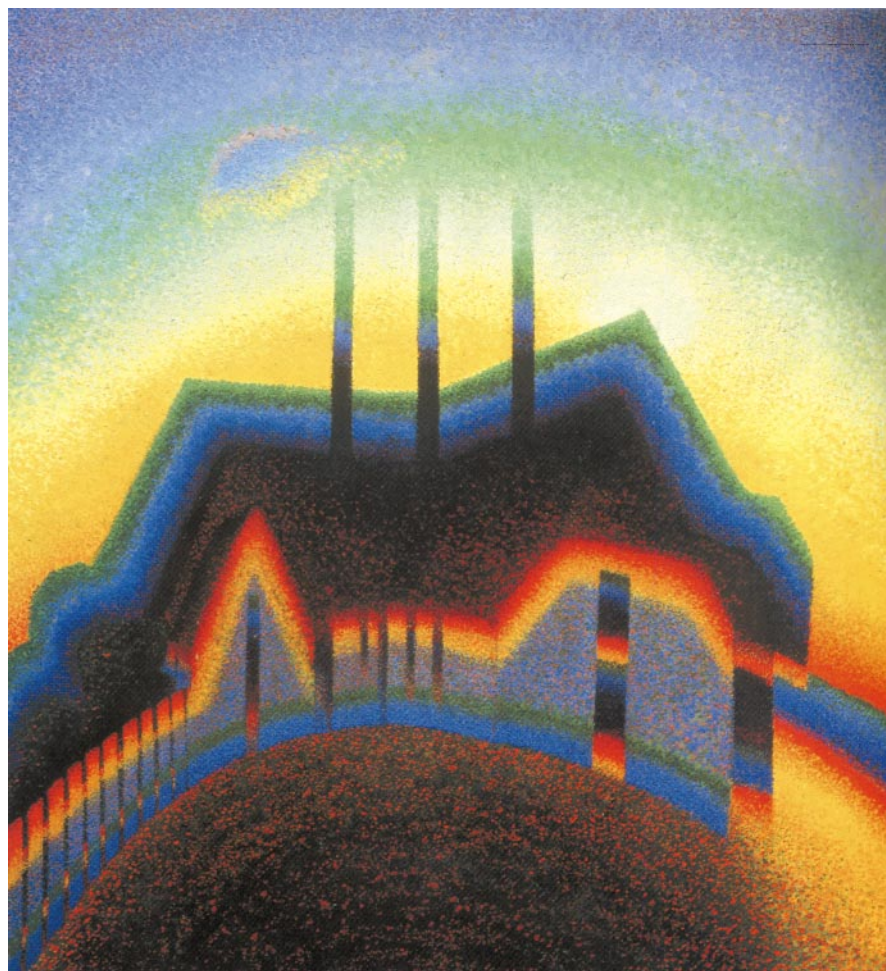
Philip Ball

"Why grass is green, or why our blood is red, Are mysteries which none have reach'd into," claimed John Donne, only to have his words superseded by Isaac Newton before the century was out. Some poets would have preferred that Donne's ignorance of colour generation remain the status quo, as John Keats expressed in his much-cited complaint that Newton's explanations would "unweave a rainbow".

Visual artists have a different attitude to science, a fact that C. P. Snow's 'two cultures' debate and its modern reincarnation unfortunately overlook. Many have struggled gamely with the sciences that inform their art, and those barriers that persist are not erected for defence but are largely the result of the sheer intellectual challenge that science presents. As the physiologist Ernst Brücke said in 1878: "Today it is difficult for the artist to be taught the theoretical science he needs, and even more difficult for him to learn it. Leonardo da Vinci was thoroughly familiar with all the knowledge of his day; he knew geometry, mechanics, physics, physiology, anatomy, all that was known of them in his time. That is impossible now because of the developments which all the sciences have undergone." The pertinence of his comment has increased over the past 120 years.

Not everyone, however, agrees that art needs science. The conservative French artist J.-G. Vibert had little time for the late nineteenth-century aspirations towards a scientific approach to coloration, lampooning the attempts of his younger contemporaries to accommodate Helmholtz and the rest. It is hard not to have some sympathy with him, when confronted on the one hand with the theorists and their dogmatic colour wheels and spheres, and on the other with the wonders wrought by Titian, Henry James's "prince of colourists", armed with only a handful of natural pigments and a master's eye for harmony and contrast.

Matisse, too, confessed: "My choice of colours does not rest on any scientific theory; it is based on observation, on feeling, on the very nature of each experience." But even he acknowledged how important scientific ideas about complementaries were to Delacroix and to the Neo-Impressionist Paul Signac, for whom "strict observation of the scientific theory of colours ... ensures a maximum of luminosity, of colour intensity, and of harmony". At least, that was the hope. In practice, both the imperfect state of scientific under-



Seeing the light: Goethe's theory of colour applied here to show the spectral edges of contrast perception.

standing of colour, and the artists' own deficiencies in comprehending what science had to tell them, led to decidedly mixed results.

It was lack of understanding, for instance, that prevented Georges Seurat's optical mixtures of pointillist dots from achieving their intended function of creating greater luminosity, instead generating a kind of greyness that even his fellow Neo-Impressionists rejected. By allowing colours to mix on the retina from closely spaced dots of complementary colours, rather than on the canvas from pigment blends, Seurat hoped to avoid the loss in brightness occasioned by subtractive mixing and to create a luminosity akin to that of sunlight dancing on reflective surfaces. But, arguably, the play of light was much better managed by Turner and later by Monet and Pissarro, who seem to have had little concern for Helmholtz's ideas on additive and subtractive mixing.

For those familiar with John Gage's masterwork, *Colour and Culture*, the obvious question is: what is left for this sequel? His perceptive analysis of Seurat's misconceptions provides one answer. But Gage comes

not to bury Seurat, as a scientific triumphalist might be tempted to. He did, after all, create some of the most haunting images in Impressionist art, and Gage is swift to point out that, by experimenting with and rejecting ideas that did not work, Seurat was not utterly removed from the scientific approach.

Although Gage is formally an art historian, the authority of his analyses stems from his being as comfortable amid Newton's *Opticks* or M. E. Chevreul's colour chemistry as he is with mediaeval iconography or the theoretical stance of the Bauhaus. Among the jewels on offer in this collection of essays on the interaction of art, art criticism, technology, physics, linguistics and much else are a discussion of Matisse's 'black light' and the link with Gustave le Bon's spurious interpretation of invisible electromagnetic radiation; a study of Wassily Kandinsky's synaesthesia in the context of the aesthetic dimension of colour; and the pre-Newtonian history of the prism. Unifying these vignettes is the relationship of the book's title: the tensions that exist between the physical processes of colour generation and attempts to find within our contingent per-

ception of it some deeper significance for the artist. Yet any quest for a universal, culturally independent interpretation of colour, says Gage, is destined to be fruitless.

But this is not a light read, even for someone moderately informed — whether scientist or artist. If you do not, for example, already have Delacroix and Ingres firmly in place as the precursors of Impressionism, this is not the book to tell you. The reader who does not know what “the ideas of Wilhelm Ostwald” on colour harmony actually were is left for more than 50 pages before being told. Cross-referencing in a book of this scope is far from trivial, especially as several chapters are compiled from articles published elsewhere. Yet this makes it all the more surprising that an editor was not on hand to excise some obvious repetitions in the initial chapters.

My only real complaint, however, is that although the book is generously endowed with high-quality colour reproduction, the very subject matter makes colour indispensable in far more cases than it is used. The monochrome illustrations are sometimes worse than useless, they are frustrating: a Turner becomes barely distinguishable from a smeared, grimy window. Obviously the economics is limiting here, but maybe electronic media could have helped?

Everything Gage writes is essential reading for the serious student of colour in art — although this book is strictly the offspring of *Colour and Culture* and you’d be well advised to read that first. *Colour and Meaning* comes as a welcome antidote to the canard that only scientists can straddle the two cultures. □

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Bedrock of geology

Lyell: The Past is the Key to the Present

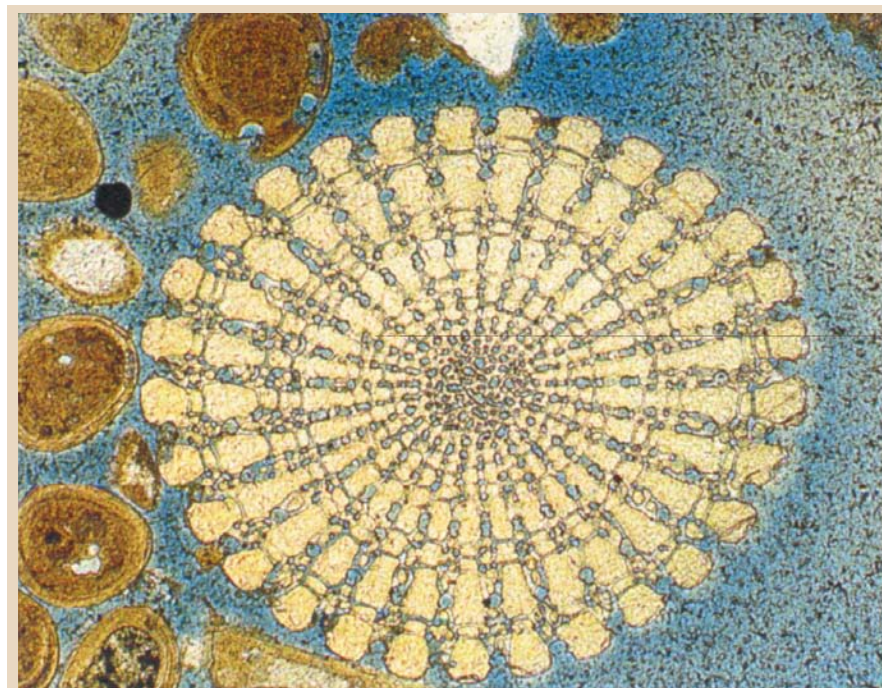
edited by D. J. Blundell and A. C. Scott
Geological Society: 1998. 376 pp. £79, \$132

Lyell in America: Transatlantic Geology, 1841–1853

by Leonard G. Wilson
Johns Hopkins University Press: 1999. 472 pp. \$45, £37

Paul Lucier

Charles Lyell (1797–1875) was one of the great scientists of the nineteenth century. He, more than anyone else, made geology a scientific discipline. Best known as the author of *Principles of Geology* (1830–1833), Lyell was the foremost advocate of what was termed uniformitarianism — the idea that the causes of geological change in the past are similar in both kind and degree to the causes of change at work today. Geologists recognize Lyell as responsible for dividing the Ter-



Patterns in the sea floor

The examination of thin sections under the microscope is a key part of any study of carbonate sediments. Intended as a laboratory manual, *A Colour Atlas of Carbonate Sediments and Rocks Under the Microscope* by A. E. Adams and W. S. MacKenzie (Manson, £24.95, pbk) contains many examples of carbonate grains and textures, such as the echinoderm spines shown here.



tiary period into Eocene, Miocene, Pliocene and Pleistocene epochs. Others might know him as Charles Darwin’s mentor. Given Lyell’s central role in the major intellectual debates of his time, it seems only fitting that we should have two celebratory books to mark the bicentenary of Lyell’s birth.

In the book edited by Blundell and Scott, we have the results of an international conference of historians and Earth scientists held in the summer of 1997 at the Geological Society of London (where Lyell himself served as secretary during 1823–26, foreign secretary in 1826, and president during 1835–36 and 1849–50) to discuss Lyell’s contributions to the geology of his own day and his influence on Earth sciences of the present.

The collection is divided into three parts. The first addresses Lyell’s life and work in geology, his travels in Europe and North America, his interactions with fellow geologists, and the impact of his ideas and discoveries on contemporaries. There are good discussions of Lyell’s reasons for writing the *Principles*, of its translation and reception in continental Europe and of the fieldwork that went into it. *Principles of Geology* was not intended as a once-and-for-all work; it was an ongoing argument that continually changed over the course of 40 years and 12 editions. During Lyell’s lifetime, some of the

ideas in the book were accepted and incorporated into standard geological practice — for example, his notion of vast amounts of time — while others were rejected. In particular, Lyell steadfastly opposed most of his contemporaries in his belief that the Earth’s history had no direction; in other words, Lyell maintained that the rock record did not reveal a progression from a hot active past to a cool quiescent present, with a broadly progressive history of life from simple plants to complex animals.

As can be seen from the subject of evolution, Lyell’s work dealt with much more than geology. The second part of the volume tries to evaluate his contributions to related scientific specialisms such as sedimentology, stratigraphy, evolution and climatic change. While many of the essays, such as a fine account of Lyell’s reception and rejection of the idea of continental glaciation, complement themes from the first part, some do not — for example, an otherwise very interesting history of hydrogeology, “a subject of passing interest to Charles Lyell”. Similarly in the third part of the volume, “The Legacy of Lyell”, studies of plate tectonics, salt tectonics or sequence stratigraphy have little

to do with Lyell, however valuable these essays are in their own right. Readers should turn to the review of research on coal or the essay on earthquakes and seismology for excellent examples of how to combine history and science.

In Leonard Wilson's book, we have a historical travelogue rather than a history of science. Wilson has given us a densely and minutely described account of the four visits (1841–42, 1845–56, 1852 and 1853) Lyell and his wife Mary made to the United States and Canada. The book is filled with the Lyells' insightful comments on American culture, customs and regional differences, such as their observations of Southern slavery and race relations. Based almost exclusively on Lyell's letters, notebooks, journals and diaries — invaluable sources to which Wilson has had unique access — it gives a tangible sense of what the Lyells experienced on their journeys: clean beds, tasty meals and congenial hosts, for the most part. But readers may start to feel suffocated by detail.

Like Wilson's first volume on Lyell's life, this is an unimaginative chronology of events. It doggedly follows Lyell between 1841 and 1853, a simplistic approach that leads to organizational imbalance. More than two-thirds of the book is devoted to a day-to-day report of the Lyells' travels in America, but in between Wilson has squeezed chapters that rush over Lyell's life between 1842 and 1845, and then between 1846 and 1852. These years were filled with travel in continental Europe, revising manuscripts (editions of the *Principles* as well as his *Elements of Geology*), delivering scientific papers and other events of greater or lesser importance, such as buying houses.

What sometimes gets lost in all the particulars is the point — geology, we assume; and Wilson does on occasion provide clear guidance on specific topics in American geology. After all, Lyell's larger purpose in going to America was to geologize. During his first two visits, he and Mary took extended tours ranging from Nova Scotia to Georgia, and from the Atlantic seaboard to the Mississippi and Ohio rivers. Lyell intended to examine the Tertiary formations and compare them with their European counterparts. He also wanted to study Niagara Falls, the Appalachians, and the coal fields and swamps of North America. Most of all, he went to collect more evidence for his theory of uniformity, which he seems to have accomplished.

Scholars, however, might question how much Lyell took versus how much he gave back. The Blundell and Scott volume includes an even-handed summary of Lyell's debt to America. Wilson, on the other hand, sees the relationship entirely through Lyell's eyes. Few scholars will see any mention of their names. Regrettably, Wilson largely ignores, or dismisses in a sharp footnote, work by fellow historians. The Blundell and Scott volume

amply demonstrates how much top-notch research has been and is being done on Lyell and nineteenth-century geology in general. Their edited volume, much more than Wilson's book, engages our interest by making the history of the Earth sciences an active part of our world today. Blundell and Scott's subtitle — *The Past is the Key to the Present* — is not only a clever reversal of the familiar uniformitarian slogan, but timely advice on how to join history and science. □

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Everyday extinctions in embattled forests

Our Forests, Our Future: World Commission on Forests and Sustainable Development

Cambridge University Press: 1999. 205 pp. £19.95, \$32.95 (pbk)

Norman Myers

As recently as 1950, forests covered 50 million square kilometres or almost 40 per cent of Earth's ice-free land. Today they cover 34 million square kilometres at most, and almost half of this has been degraded from its primary form. This is far and away the biggest land-use change to overtake the planet in such a short space of time.

We shall pay heavily for what is lost already (developing nations forgo \$45 billion per year through poor forest management), let alone what will be eliminated if we allow present exploitation patterns to persist. At the current rate of deforestation, we are likely to witness the demise of most tropical forests within the lifetimes of most readers of this journal. In addition, global warming, with its shifts in temperature and precipitation bands, may well cause an extensive die-back of boreal forests.

As this book demonstrates at length, forests are fundamental to our biosphere's workings. They provide commercial timber, fuel and many non-wood products. Tropical forests alone contain more than half of all species and, because some 160,000 square kilometres of them are destroyed every year, they are the site of most extinctions. Some 30,000 plant and animal species out of a planetary spectrum of 10 million become extinct every year.

Still more valuable are forests' environmental services. They protect soils with their moisture and nutrients. They protect watersheds and thus regulate water flows in quantity and quality. They modulate climate at local and regional levels through regulation of rainfall regimes and the albedo effect.

They help to slow global warming by virtue of their carbon sinks with 1,200 giga-

tonnes out of 2,000 gigatonnes of carbon in all terrestrial plants and soils. At present rates of burning (1998 saw more forests sent up in smoke than ever before, not just in Borneo and Amazonia but in Canada and Siberia as well), they release perhaps 1.3 gigatonnes of carbon per year, or one-fifth as much as emitted by combustion of fossil fuels. Conversely, forests absorb carbon dioxide from the global atmosphere, and the Northern Hemisphere's forests may be sequestering a large but indeterminate amount of carbon. A salient way to resist global warming would be through grand-scale reforestation of the humid tropics, where trees grow several times faster than in, say, Britain.

Despite their many benefits, forests rank among the least developed of all natural resources. They are still not used in the sustainable ways that could provide both material goods and environmental services, while serving the long-term interests of all communities concerned. Many forests are exploited for only a few outputs, notably timber and agriculture, with adverse consequences for their many other actual or potential outputs. They are both over-exploited and under-utilized.

We should therefore welcome this report of the World Commission on Forests and Sustainable Development. An attempt to tackle the decline of forests was broached at the Rio Earth Summit, all the more pertinent in light of forests' many linkages to the Climate and Biodiversity Conventions achieved at Rio. Alas, the effort foundered on the politics of sovereignty: all forests fall within the jurisdiction of individual nations, and many governments, notably that of Brazil (with two-fifths of tropical forests), were not willing to cede any element of perceived control to a global institution.

This was the rationale for the World Commission's attempt to take another crack at one of the great issues of our time: "The decline of forests could change the very character of the planet and the human enterprise within a few years unless we make some choices." These choices include "radical reform, a new political agenda, greater civil society involvement, and more science in policy making".

Fortunately the book recognizes a key constraint: that forestry policy is not generally set by foresters, however much they may believe the contrary. Forests are largely eliminated not by over-logging but by agricultural encroachment, industrial pollution and a host of other non-forestry factors. To save the day, there is urgent need for (a) a greater appreciation by governments of the full panoply of forest values, and (b) a more vigorous assertion by foresters of what is at stake for activities and sectors far beyond forests. To the book's credit, this point is hammered home time and again.

This is a fine book, which reviews the

present status and future outlook of forests comprehensively, with stacks of ecological and economic statistics and broad-scope analyses. Yet it could have been more adventurous. It largely supposes that forests' outputs will remain an extension of the past. What if China's construction boom were to cause its per-capita consumption of two main timber products, sawn wood and panels, to triple and match Indonesia's (not a high level)? China would become the world's leading importer of wood — and impose pressures on foreign forests far outstripping Japan's over the past several decades.

What, too, if certain wood applications were to be partially overtaken by substitutes for, say, paper pulp, as is already the case for 90 per cent of China's feedstocks, where rice straw and sugar-cane bagasse have been substituted for pulp feedstocks? What if many uses of construction timber were to give way to substitutes such as alloys and composites, as is already the case in many developing countries, where people use mud bricks reinforced with chopped sisal or elephant grass? According to a report by the UN Centre for Human Settlements (*An Urbanizing World: Global Report on Human Settlements*, 1996, Nairobi, Kenya), within the next

decade, developing countries will need to build at least 250 million new dwellings, or half as many as have been built since we came out of our caves. What if biotechnology were to enable us to grow square trees (along the lines of the square tomatoes we already have), providing twice as much board timber as round trees? □

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Experimenting with drugs

Instant Pharmacology

by Kourosh Saeb-Parsy, Ravi G Assomull, Fakhar Z. Khan, Kasra Saeb-Parsy and Eamonn Kelly

Wiley: 1999. 349 pp. \$34.95, £19.99 (pbk)

John Foreman

Writers of undergraduate medical textbooks have a knowledge and enthusiasm for their subject that often results in a massive book, intimidating students with its size, depth and breadth. *Instant Pharmacology* is a novel departure from this because its

writers are students themselves.

The book also departs from the standard format of pharmacology texts. The first section, which describes the basic principles of drug action, is followed by sections on the principles of chemical transmission and the mechanisms of drug action on the different body systems. Next comes a dictionary of drugs, set out in alphabetical order of generic drug name. The final section is a set of questions and answers designed to aid self-directed learning.

The book is appealing because it is concise and shorter than many of the current major textbooks. The dictionary of drugs is a useful way of locating information on a particular drug. The format lends itself to the systems-based approach to medicine that many medical schools are now adopting, and the actions of drugs are successfully placed in the relevant clinical contexts. No doubt, students will like the idea that their peers have written the text with a view to removing all that is not core material.

Some problems, however, arise from the novel format and the content. The text contains features that are likely to mislead or confuse the student reader. For example, the peptide hormones angiotensin and bradykinin have been classified as neuro-peptides, and the duration of warfarin's action is attributed to the rate of catabolism of clotting factors rather than to the half-life of the drug.

One of the problems that students new to pharmacology encounter is the sheer volume of drug names. An introductory text should concentrate on mechanisms of action and provide only main examples of each type or sub-type of mechanism. But *Instant Pharmacology* has numerous examples of each mechanism in some areas and few in others.

There are tensions between the main text and the dictionary. The main text is not very informative about the mechanisms of action of some drug classes, and the dictionary contains information that more logically would have been placed in the text, since it relates to the mechanism of action. In other cases, the text is merely repeated in the dictionary.

There is a place in the market for a concise introductory pharmacology text. It remains to be seen whether students will appreciate the novel approach in this book. □

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New in paperback

Forbidden Drugs, 2nd edn

by Philip Robson

Oxford University Press, £12.99



Lunar celebration

Marking the 30th anniversary of the first landing on the Moon, *Full Moon* (Jonathan Cape, £35) is a photographic journey to the Moon and back, gleaned from NASA's 32,000 pictures from the Apollo space missions.

Photographer Michael Light has used 'master' negatives from NASA's collection to create a pictorial narrative of the launch, the walk in space, the landing and the return to Earth. The book also contains an essay by Andrew Chaikin.

Neural correlates of decision variables in parietal cortex

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Decision theory proposes that humans and animals decide what to do in a given situation by assessing the relative value of each possible response. This assessment can be computed, in part, from the probability that each action will result in a gain and the magnitude of the gain expected. Here we show that the gain (or reward) a monkey can expect to realize from an eye-movement response modulates the activity of neurons in the lateral intraparietal area, an area of primate cortex that is thought to transform visual signals into eye-movement commands. We also show that the activity of these neurons is sensitive to the probability that a particular response will result in a gain. When animals can choose freely between two alternative responses, the choices subjects make and neuronal activation in this area are both correlated with the relative amount of gain that the animal can expect from each response. Our data indicate that a decision-theoretic model may provide a powerful new framework for studying the neural processes that intervene between sensation and action.

Neurobiologists have begun to focus increasingly on the study of sensory–motor processing, but many of the models used to describe these processes remain rooted in the classic reflex, initially described by Descartes¹ and later advocated by Sherrington² as a model system for studying the connection between sensation and movement. Sherrington, in particular, viewed response selection as a physical link between independent, anatomically distinct sensory and motor systems². Until recently, the sensory–motor reflex was tacitly accepted by many physiologists as an appropriate model for describing the neural processes that underlie complex behaviour^{3–5}. More recent findings indicate that, at least in some cases, the neural events that connect sensation and movement may involve processes other than classical reflexive mechanisms^{6–9} and these data support theoretical approaches that have challenged reflex models directly^{10–13}. Researchers outside physiology have long argued for richer models of the sensory–motor process^{14–18}. These models invoke the explicit representation of a class of decision variables, which carry information about the environment, are extracted in advance of response selection, aid in the interpretation or processing of sensory data and are a prerequisite for rational decision making¹⁹. Here we describe a formal economic–mathematical approach for the physiological study of the sensory–motor process, or decision-making, in the lateral intra-parietal area (LIP) of the macaque brain.

Theoretical treatments of decision making

Although mathematical approaches to decision-making propose different formulae for identifying the response a subject should choose under a given set of conditions, nearly all theories require the decision-maker to have some knowledge of two environmental variables: the gain expected to result from an action and the probability that the expected gain will be realized. The expected value theory of Arnaud and Nichol²⁰, for example, proposes that a rational decision-maker should multiply expected gain by the probability of gain to establish an expected value for each course of action, and then should choose the option with the highest expected value. Although subsequent theorists have proposed different mathematical combinatorial rules that provide more accurate models of the decision-making process, knowledge of the gain expected from a response and the probability of realizing that gain is still considered to be critical to the computation of rational choice^{19,20,22}.

Ecological biologists^{17,18} and psychologists^{14–16} have also developed formal models of decision-making. Most of these models

assume that animals make decisions that either maximize the rate of energy intake from different options or match their rates of behavioural responding to the reinforcement rates obtained from different choices. These models propose that animals choose on the basis of several variables, including the probability of reinforcement²³ and the magnitude of reinforcement^{24,25} associated with different responses. Experimental evidence indicates that expected gain and probability of gain do influence the choices animals make.

However, neurobiological models of the processes that connect sensation and action almost never propose the explicit representation of decision variables by the nervous system. Most contemporary neurophysiological models, particularly models of cortical sensory–motor transformations, identify sensory and motor signals as critical components, but they rarely address the possibility that neural signals may encode data that fall outside these two categories. Our investigations have led us to propose that decision theory might provide a powerful alternative framework for studying the sensory–motor process. Our current model could be described as having two classes of inputs: current sensory data, and a stored representation of environmental contingencies. Current sensory data would reflect the observer's best estimate of the current state of the salient elements of the environment. As such, it would be influenced by stored information that could improve the efficiency of sensory processing through selective attention. The second class of inputs would represent the chooser's assumptions about current environmental contingencies, a class of inputs that detail how an action affects the chooser. Decision making would involve the combination of post-attentional sensory data with the subject's best estimate of the outcome of any given action. These estimates of environmental contingencies would then be combined with a loss function specifying the value of all possible losses and gains to the chooser, according to a decision rule like the one originally proposed by Arnaud and Nichol²⁰ or in subsequent refinements of their model^{19,21,22}.

Physiology of sensory–motor integration in LIP

Although neurobiologists have not employed decision-theoretic formulations of this type, many groups have concluded that a number of brain areas are involved in the processes that intervene between sensation and action. Parietal area LIP has been labelled attentional^{26,27}, decision-related²⁸ and motor-preparatory^{29–31} by different groups. Our own work indicates that decision-theoretic models may provide a powerful alternative approach to the study of sensory–motor processing in parietal cortex^{32,33}.

In one study³⁴, we assumed that visual attention can be treated as conceptually separable from other elements of the decision-making process. We used two tasks that independently controlled both the location of a saccadic target and the location and behavioural relevance of a visual distractor. In both tasks, a monkey initially fixated a central stimulus, and then two eccentric stimuli were illuminated, one above and one below the central stimulus. A change in the colour of the central stimulus identified one eccentric stimulus as the eventual saccadic target and the other as a visual distractor. In one task, the distractor was highly relevant because

offset of this stimulus signalled the animal to initiate a saccade to the target; in the second task the distractor was completely irrelevant, because offset of the central stimulus signalled the animal to initiate the saccade. Although intraparietal neurons were more active on blocks of trials in which a stimulus served as a saccadic target than on blocks of trials in which the same stimulus served as a visual distractor, none of the neuronal responses distinguished between behaviourally relevant and irrelevant distractors. We took this finding as preliminary evidence that activity in area LIP does not participate in sensory-attentional processing but is correlated with either outcome contingencies, gain functions, decision outputs or motor planning, all processes that our decision-theoretic approach indicates are critical in choice behaviour.

Experimental design

Here we examine whether a decision-theoretic model might reveal a link between the activity of area LIP neurons and the choices animals make in an oculomotor task. In the first experiment (experiment 1; Figs 1, 2), we monitored the activity of single intraparietal neurons while animals performed cued saccade trials, in which a change in the colour of a centrally located fixation stimulus instructed subjects to make one of two possible eye-movement responses in order to receive a juice reward. Before this stimulus changed colour, the rewarded movement was ambiguous. In successive blocks of trials, we varied either the volume of juice delivered for each instructed response (expected gain) or the probability that each possible response would be instructed (outcome probability), while holding all sensory signals and motor-related variables constant. This allowed us to test whether any portion of the variation in area LIP spike rates observed under these conditions was correlated with variations in the decision variables we manipulated.

In the second experiment (experiment 2; Figs 3, 4), animals were rewarded for choosing either of two possible eye-movement responses. In sequential blocks of trials, we varied the gain that could be expected from each possible response and then used the frequency with which the animal chose each response as an estimate of the subjective value of each option. This permitted us to test whether neural activity in area LIP was correlated with a behaviourally derived estimate of response value. Our data indicate that some of the variation in the LIP activity was, in fact, correlated with economic decision variables and that these variables influenced the choices made by our animal subjects and neural activity in a similar manner.

Decision variables and LIP spike rates

Figure 1a shows the activity of an intraparietal neuron on visually identical cued saccade trials during which the monkey was instructed to shift gaze into the neuronal response field for a juice reward. When, in one block of trials (thick black line), 0.26 ml of juice was delivered, this neuron was more active than when the same movement was made in response to the same visual display but during a block (thick grey line) in which only 0.09 ml of juice was delivered. This neuron was studied while expected gain was systematically varied across seven blocks of trials, and behavioural performance was maintained at 91% correct. To quantify the effects of these manipulations on the activity of this neuron, we computed average firing rates in six 200-ms epochs during each trial (early visual: 0–200 ms after the onset of the two response targets; late visual: 200–0 ms before the central light-emitting diode (LED) changed colour; early cue: 0–200 ms after the central LED identified the movement that would be reinforced; late cue: 200–0 ms before the command to initiate the movement; pre-movement: 0–200 ms before saccade onset; post-movement: 0–200 ms after saccade onset). Figure 1b shows the firing rate of this neuron (mean $\pm$ s.e.) as a function of the gain (in ml of juice) expected for a movement into the response field, divided by the sum of the

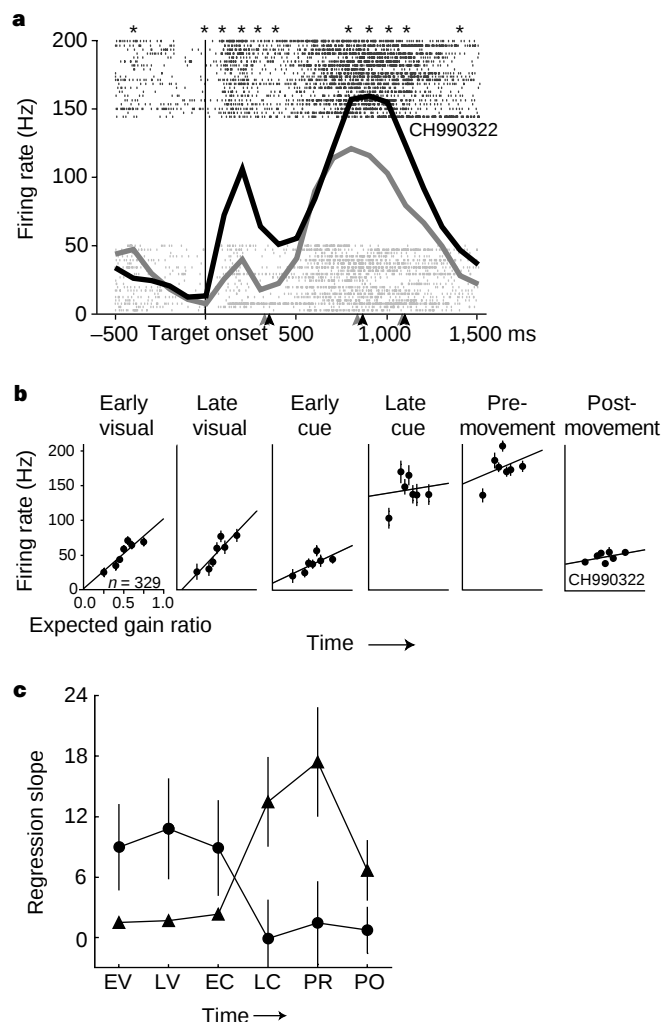


Figure 1 Modulation of neuronal activity by expected gain. The volume of juice delivered for each response was varied across blocks of cued saccade trials.

a, Firing rate of an intraparietal neuron on trials instructing gaze shifts into the response field, averaged in 100 ms bins and synchronized, at vertical line, on target onset. Thick black line, expected gain ratio = 0.75 ($n = 48$); thick grey line, expected gain ratio = 0.25 ($n = 37$). Raster panels show spike times during the first 20 high-gain (dark grey) and first 20 low gain (light grey) trials. Panels show individual trials in sequential order from top to bottom. Arrows plot, successively, mean times of instruction cue onset, central fixation stimulus offset and saccade onset during high- (black arrow) and low- (grey arrow) gain blocks. Stars indicate 100-ms bins in which firing rate was significantly different in the high-gain block than in the low-gain block (t -test, $P < 0.05$). **b**, Mean firing rate ($\pm$ s.e.) during each interval plotted against expected gain ratio, for all trials instructing gaze shifts into the neuronal response field. Lines indicate best-fit linear regressions through the raw data. **c**, Mean ($\pm$ s.e.) regression slope for expected gain (circles) and the instructed movement (triangles), plotted against time ($n = 40$ neurons). Intervals: EV, early visual; LV, late visual; EC, early cue; LC, late cue; PR, pre-movement; PO, post-movement.

Table 1 Percentages of recorded neurons showing significant correlations between firing rate and expected gain, outcome probability or estimated value

Decision variable	Early visual/fixation*	Late visual/fixation*	Early cue/visual*	Late cue/visual*	Pre-movement	Post-movement	Total
Expected gain	27.5% (17.5%)	32.5% (15%)	40% (27.5%)	27.5% (62.5%)	30% (72.5%)	15% (65%)	62.5% (87.5%)
Outcome probability	35% (10%)	35% (10%)	40% (40%)	15% (100%)	30% (95%)	15% (95%)	75% (100%)
Estimated value	28% (6%)	17% (28%)	28% (47%)	19% (67%)	19% (67%)	25% (69%)	56% (89%)

Each entry indicates the percentage of cells in each experiment showing significant ($P < 0.05$) modulations in firing rate during each interval. Entries in parentheses indicate the percentage of cells that showed significant modulations in firing rate by the movement actually made by the animal. Total percentages indicate the percentage of neurons showing a significant modulation in firing rate during any interval. Asterisks identify the names of intervals measured in experiment 2 (early fixation, late fixation, early visual, late visual).

gain (in ml) available from both possible movements, for all those trials in which the animal shifted gaze to the target inside the response field of the neuron. The firing rate of this intraparietal neuron was correlated with this expected gain ratio early in the trials (early visual, $r = 0.432$, $P < 0.0001$; late visual, $r = 0.372$,

$P < 0.0001$; early cue, $r = 0.217$, $P < 0.0001$), but later in the trials, around the time of the movement, firing rate and expected gain ratio were uncorrelated. These data indicate a temporal shift in the information encoded by this neuron, from expected gain early in trials to the movement made by the animal at the end of trials. On these same trials, movement amplitude ($r = -0.055$, $P > 0.322$), movement latency ($r = 0.016$, $P > 0.767$) and movement velocity ($r = 0.02$, $P > 0.718$) were uncorrelated with expected gain.

Each of 40 intraparietal neurons recorded from the brains of three monkeys was tested in 3–7 blocks of trials (mean = 4.7) in which the gain associated with each of the two target LEDs was different. For each neuron, correct trials were sorted into two groups based on whether the cued movement was into or out of the response field. For all trials in which the subject shifted gaze towards the response-field target, we analysed the relationship between firing rates computed during each interval described above and four variables: expected gain; the amplitude of each saccade; the average velocity of each saccade; and the latency of each saccade. We used a multiple regression analysis to determine which portion of firing-rate modulation could be uniquely attributed to changes in expected gain independently of changes in any of the three remaining movement-related variables. For comparison, a second multiple regression analysis was performed to determine the degree to which the firing rate of each neuron was uniquely influenced by which movement the subject was cued to make, after correcting for the effects of expected gain, movement amplitude, movement latency and movement velocity.

The slopes generated by the multiple regression analyses were used as an index of how strongly changes in expected gain modulated the activation of intraparietal neurons, independently of changes in movement amplitude, latency and velocity. Figure 1c shows the mean regression slope ($\pm$ s.e.) for 40 intraparietal neurons for expected gain (circles); the mean slopes for the instructed movement (triangles) are provided for comparison. The regression slopes for expected gain were significantly greater than zero (single-sample t -test, $P < 0.05$) during the early visual and late visual intervals; expected gain strongly modulated the activation of the population of intraparietal neurons early in trials, before the identification of the rewarded movement. After the rewarded movement was identified, however, the activation of the intraparietal population was strongly modulated by the instructed movement, but not by expected gain.

Table 1 shows the percentages of neurons in this sample that were significantly modulated by expected gain during each interval, with the percentages of neurons significantly modulated by the instructed movement in each interval provided in parentheses for comparison. Overall, 62.5% of parietal neurons carried statistically significant information about expected gain at some point during the trial, and 87.5% of neurons carried statistically significant information about which movement was actually made. These data indicate that the activation of the intraparietal neuronal population is correlated with expected gain ratio, particularly during periods of uncertainty early in trials when decision variables might be important in formulating a movement plan.

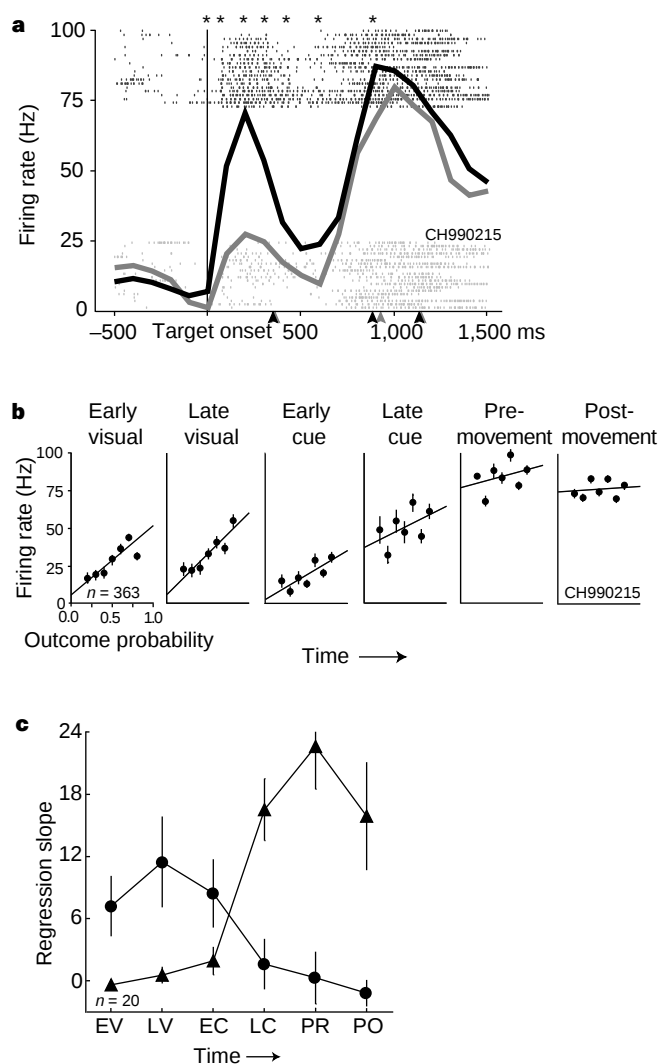


Figure 2 Modulation of neuronal activity by outcome probability. The probability that each response would be instructed was varied across blocks of cued saccade trials. **a**, Firing rate of an intraparietal neuron on trials instructing a gaze shift into the response field, as in Fig. 1. Thick black line, outcome probability = 0.80 ($n = 77$); thick grey line, outcome probability = 0.20 ($n = 26$). **b**, Mean firing rate ($\pm$ s.e.) during each interval plotted against outcome probability, for all trials instructing gaze shifts into the neuronal response field. **c**, Mean ($\pm$ s.e.) regression slope for outcome probability (circles) and the instructed movement (triangles) plotted against time ($n = 20$ neurons).

Figure 2a shows the modulation of the activity of an intraparietal neuron by changes in outcome probability during visually identical cued saccade trials. When a saccade into the response field was instructed with a probability of 0.8 (thick black line), the neuron was more active than when an equivalent movement was instructed with a probability of 0.2 (thick grey line). This neuron was studied while outcome probability was varied across seven blocks of trials, and behavioural performance was maintained at 85% correct. Figure 2b shows the firing rate of this neuron (mean $\pm$ s.e.) as a function of outcome probability during each of the six measured epochs, for all trials on which the animal was instructed to shift gaze to the response field of the neuron. During the early visual ($r = 0.407$, $P < 0.0001$), late visual ($r = 0.349$, $P < 0.0001$), early cue ($r = 0.242$, $P < 0.0001$), late cue ($r = 0.131$, $P < 0.012$) and pre-movement intervals ($r = 0.116$, $P < 0.027$), firing rate increased with increases in outcome probability. Movement amplitude ($r = 0.056$, $P > 0.291$), latency ($r = -0.098$, $P > 0.061$) and velocity ($r = 0.07$, $P > 0.183$) were not correlated with outcome probability. The activity of this neuron was thus correlated with the probability that the animal would be instructed to choose each response, independent of the movement actually made by the animal.

We studied the effects of varying outcome probability across 5–7 blocks of trials (mean = 6.7) on 20 intraparietal neurons in two monkeys. Overall, 75% of the intraparietal neurons in our sample carried significant information about outcome probability at some point during the trial, while all 20 neurons carried significant information about which movement was actually made (Table 1). Figure 2c shows the mean ($\pm$ s.e.) regression slopes for outcome probability (filled circles) and the instructed movement (filled triangles) for our population. The regression slopes for outcome probability were significantly greater than zero during the early visual, late visual and early cue intervals (single-sample t -tests, $P < 0.05$). The movement made by the animal significantly modulated neuronal activity in this population only after the cue identified the reinforced movement (late cue, pre-movement and post-movement intervals, single-sample t -tests, $P < 0.05$). The modulations induced by changes in outcome probability were very similar to those evoked by changes in expected gain and support the idea that outcome probability influences decision processes before the colour of the fixation stimulus identifies the reinforced movement.

Response value and LIP neural activity

The results of experiment 1 show that changes in either the gain expected from a particular response or the probability that a particular response will be required modulate the activity of intraparietal neurons. This is true even when all the visual and motor events associated with each trial are the same. In experiment 1, however, we were limited in our ability to assess the effects of expected gain and outcome probability on decision-making because there was a single correct choice identified in each trial, making it difficult to correlate any aspect of the choices made by our animal subjects with neuronal activity. In experiment 2, we therefore omitted the centrally located colour cue that identified only one of the responses as reinforced and instead allowed subjects to choose either of the two responses while we systematically varied the gain that could be expected from each response across 5–7 blocks of trials (mean, 5.8). The actual choices made by subjects were then used as an estimate of the valuation of each response by the animal on each trial and neuronal data was related directly to this behavioural readout of the animal's decision process.

Figure 3a shows, for visually identical trials in which the animal chose to shift gaze into the response field of the neuron under study, the modulation in activity of an intraparietal neuron in this free-choice task. The neuron was more active during a block of trials rewarded with 0.15 ml of juice (Fig. 3a, thick black line) than in

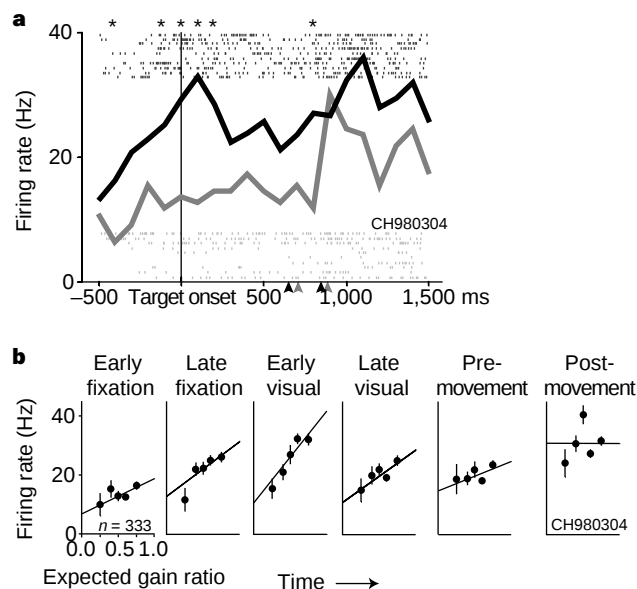


Figure 3 Modulation of the activity of a single intraparietal neuron by expected gain in a free-choice task. The volume of juice delivered for each response was varied across blocks of saccadic choice trials. **a**, Firing rate of an intraparietal neuron on trials in which subjects chose to shift gaze into the response field, as in Fig. 1. Thick black line, expected gain ratio = 0.75 ($n = 75$); thick grey line, expected gain ratio = 0.25 ($n = 11$). Raster panels plot spike times during the first 10 trials in both the high-gain (dark grey) and low-gain (light grey) blocks. Arrows plot, sequentially, the mean times of central fixation stimulus offset and saccade onset in high- (black arrows) and low- (grey arrows) gain blocks. **b**, Mean firing rate ($\pm$ s.e.) during each interval plotted against expected gain, for movements into the response field.

another block of trials in which the same movement was rewarded with only 0.05 ml of juice (Fig. 3a, thick grey line). In fact, during the high-gain block the neuron became activated before the onset of the two choice targets, a pattern of activation that cannot be attributed to a visual response to target onset.

We also measured the average firing rate of this neuron during six 200-ms epochs (early fixation, late fixation, early visual, late visual, pre-movement and post-movement). Figure 3b shows the firing rate of this neuron (mean $\pm$ s.e.) during these epochs (note that two of these intervals, early fixation and late fixation, are before the illumination of the visual targets). Firing rate increased with expected gain during the early fixation ($r = 0.158$, $P < 0.025$), late fixation ($r = 0.181$, $P < 0.01$), early visual ($r = 0.263$, $P < 0.001$) and late visual ($r = 0.187$, $P < 0.01$) intervals, but not around the time of the movement.

These data confirm the correlation between neuronal activation and the gain that could be expected from a particular movement, as reported in the previous experiments. The goal of this experiment, however, was to directly correlate neuronal activity with the animal's estimate of the value of the two possible movements. Figure 4a presents the choices the subject made across all blocks of trials during this recording session. Consistent with Herrnstein's matching law¹⁴ for choice behaviour, there was a linear relationship between the proportion of trials on which the animal chose the target inside the response field and the proportion of total juice available for gaze shifts to that target ($r = 0.96$, $P < 0.008$)^{35,36}. Across 36 data sets, there was a high correlation between the frequency with which monkeys shifted gaze to a target and the proportion of total juice available for gaze shifts to that target (mean $r = 0.89$, mean $P = 0.03$, mean slope = 1.86, mean intercept = -0.45). Thus, the choice behaviour of our subjects reliably reflected the gain

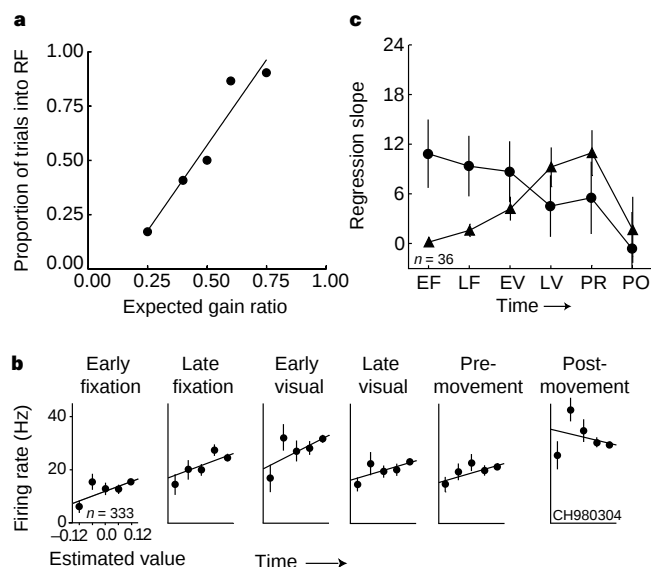


Figure 4 Correlation of firing rate with a behavioural estimate of the subjective value of the two movements. **a**, Proportion of trials on which the monkey chose the target inside the neuronal response field plotted against expected gain. Line indicates best-fit linear regression. **b**, Mean firing rate ($\pm$ s.e.) during each interval plotted against a behavioural estimate of the subjective value of each response, computed on each trial from the difference in the reinforcement rates obtained from the two possible movements during the preceding 10 trials²⁴. **c**, Mean ($\pm$ s.e.) regression slope for estimated value (filled circles) and the chosen movement (filled triangles) plotted against time ($n = 36$ neurons). Intervals as in Fig. 1c, except: EF, early fixation; LF, late fixation.

associated with each movement. In subsequent analyses, we therefore used choice frequencies as an estimate of the subject's relative valuation of the two reinforced responses.

To analyse the relationship between the trial-by-trial activity of this neuron and the valuation of each choice by the subject, on each trial we computed a behavioural estimate of the subjective value of a movement into the response field, based on Herrnstein's melioration theory²³, by computing the difference in the rate of reinforcement the animal had obtained from each of the two possible choices over the preceding 10 trials (estimated value). Figure 4b shows the mean firing rate of the neuron as a function of this estimated value, during each measured interval, for all trials on which the animal shifted gaze into the response field. The firing rate of this neuron increased as the estimated value of a movement into the response field increased during the early fixation ($r = 0.204$, $P < 0.004$), late fixation ($r = 0.152$, $P < 0.031$), early visual ($r = 0.180$, $P < 0.01$) and pre-movement ($r = 0.144$, $P < 0.04$) intervals. The firing rate of this neuron was uncorrelated with saccade amplitude, velocity and latency during all six measured intervals, and thus these motor variables cannot account for the relationship between firing rate and the subjective value of a movement into the response field.

We recorded the activity of 36 intraparietal neurons from two monkeys performing this free-choice task. Overall, the regression slopes for estimated value were significantly greater than zero, at some point during trials, for 56% of the neurons in the parietal population, while the regression slopes for the chosen movement were significantly greater than zero for 89% of the population (Table 1). Figure 4c shows the mean ($\pm$ s.e.) regression slope for the estimated value of a movement into the response field (circles) during each epoch, as well as the mean ($\pm$ s.e.) regression slope for the chosen movement (triangles). The estimated value of a movement into the response field significantly modulated activity in our intraparietal neuronal population during the early fixation, late

fixation and early visual epochs (single-sample t -tests, $P < 0.05$). The movement chosen by the animal significantly modulated activity in this same population of neurons during the late fixation, early visual, late visual and pre-movement epochs. Thus, when an animal is presented with a choice between two possible saccadic gaze shifts, the animal's estimate of the relative value of these two movements is correlated with the activation of intraparietal neurons. The activation of intraparietal cortex therefore appears to reflect the decision processes animals use to guide behaviour.

Discussion

Our results indicate that a decision-theoretic model of the sensory-motor process may provide a powerful alternative approach to traditional sensory and motor neurophysiological models of response selection. Experiment 1 indicates that both the gain expected from a particular response and the probability that a particular response will be required systematically increase the activation of neurons in posterior parietal cortex. Furthermore, this influence is separable from the effects of the immediate visual environment and from the neural events that govern movement dynamics. The effects of expected gain and outcome probability were strongest before the time at which the animal knew which of the two possible responses would be rewarded. Experiment 2 shows that when an animal is free to choose between alternative responses, the gain expected from each possible action exerts a correlated influence on both the choice behaviour of the animal and the activation of posterior parietal neurons. In our free-choice task, both monkeys and posterior parietal neurons behaved as if they had knowledge of the gains associated with different actions. These findings support the hypothesis that the variables that have been identified by economists, psychologists and ecologists as important in decision-making are represented in the nervous system.

Our data are, however, difficult to reconcile with traditional sensory-attentional or motor-physiological models. For example, sensory-attentional models typically attribute modulations in the activity of visually responsive neurons to changes in the behavioural relevance of visual stimuli²⁶ and, based on these models, it could be argued that our manipulations of expected gain and outcome probability merely altered the visual activity of intraparietal neurons. This interpretation seems unlikely for two reasons. First, when an animal is instructed to plan a gaze shift to one of two simultaneously presented eccentric visual stimuli, intraparietal neurons are insensitive to changes in the behavioural relevance of either stimulus when it is not the target of a saccade³⁴. Second, in experiment 2 we showed that intraparietal neuronal activity was modulated by changes in the estimated value of each response during the fixation intervals before the onset of the response targets, when subjects were fixating the central LED in otherwise total darkness. We find this pattern of activation difficult to attribute to an attentional modulation of visually evoked activity.

It might also be suggested that the modulations in neuronal activity we evoked by changes in expected gain, outcome probability and estimated value reflect changes in the degree to which animals plan particular movements. Choice behaviour, such as that shown by subjects in experiment 2, might thus reflect the outcome of a covert competition between movement plans weighted by the estimated value of each possible action. However, because such a model must include an estimate of response value it is essentially equivalent to a decision-theoretic model.

Although the decision variables we have examined here systematically modulated the activity of intraparietal neurons in our tasks, these effects were, for most neurons, confined to the early portions of each trial. Late in each trial, intraparietal neurons were more strongly modulated by the animal's movement plan than by expected gain, outcome probability or the animal's estimate of response value. This may indicate that intraparietal cortex lies close to the motor output stages of the sensory-decision-motor process.

These data may also indicate that other brain areas believed to participate in decision-making, such as prefrontal cortex³⁷, might represent decision variables throughout the trial.

In conclusion, our results indicate that a neurobiological framework for the study of choice based upon the same classical decision theory that has guided behavioural studies of the choices humans and animals make may provide a powerful paradigm for studying the sensory–decision–motor process. Moreover, this framework indicates that many of the modulatory influences attributed to sensory attention or motor planning might be more precisely described as a reflection of decision processes. The influence of these variables on the activity of posterior parietal cortex, a brain area believed to participate in the transformation of sensory signals into motor commands, indicates that a decision-theoretic framework might be a useful tool for understanding the neurophysiology of sensory-guided behaviour. □

Methods

Single intraparietal neurons were studied in the brains of three rhesus monkeys trained to perform eye movements, in response to visual cues, for a fruit juice reward. Standard electrophysiological, behavioural and histological techniques were employed, as described³⁴. All procedures were approved by the New York University Animal Care and Use Committee and were in compliance with the Public Health Service's *Guide for the Care and Use of Animals*.

Each experiment began with the electrophysiological isolation of a single intraparietal neuron. Animals were then instructed to make a series of 50–200 eye movements from a central starting position to successively illuminated LEDs randomly selected from among several hundred LEDs located at different peripheral positions. We used these movements to identify an eccentric LED that elicited a movement for which the neuron was maximally active (a movement into the response field) and an eccentric LED that elicited a movement for which the neuron was minimally active (a movement out of the response field).

Experiment 1. For experiment 1, this mapping procedure was followed by a series of cued saccade trials in total darkness. Each of these trials began with the illumination of a yellow LED located directly in front of the animal. A random interval (200–500 ms) after the subject had aligned his gaze ($\pm 2^\circ$) with this LED, the two eccentric yellow response LEDs identified by our mapping procedure were illuminated. After another random interval (200–800 ms), the central LED changed colour to either red or green, instructing the subject that a saccade aligning gaze with either the upper or lower eccentric LED, respectively, would be rewarded with a drop of juice. After a final random interval (200–800 ms), the central LED was extinguished and the monkey was rewarded if he shifted gaze into alignment with ($\pm 4^\circ$) the correct eccentric LED. We then determined whether the activation of intraparietal neurons was modulated by systematic changes in the gain expected from each possible movement or the probability that each of the two possible movements would be instructed. Animals typically performed this task correctly on about 90% of trials.

Expected gain. While each of 40 neurons was studied we varied, in 3–7 sequential blocks of about 100 trials each, the amount of juice reward the animals received both for movements into and movements out of the response field (expected gain). Across blocks, the sum of the juice available from these two possible movements was held constant, and the probability that either response would be instructed on a given trial (outcome probability) was held constant at 0.5. An experiment typically began with a block in which each movement was reinforced equally, and then pairs of blocks of complementary high gain/low gain conditions were randomly interleaved.

Outcome probability. While each of 20 neurons was studied we varied, in 5–7 sequential blocks of about 100 trials each, the probability that each of the two responses would be instructed while the gain expected from each movement was held constant. A block in which each movement was instructed equally often was typically run first, and then pairs of blocks with complementary high probability/low probability conditions were randomly interleaved.

Experiment 2. Thirty-six neurons were studied in two rhesus monkeys performing saccadic choice trials, which were similar to cued saccade trials but had no centrally located colour cue. Each of these trials began with the

illumination of a yellow LED located directly in front of the animal. 500 ms after the subject had aligned his gaze ($\pm 2^\circ$) with this LED, the two eccentric yellow response LEDs identified by our mapping procedure were illuminated. After a random interval (500–800 ms), the central LED was extinguished and the monkey was rewarded if he shifted gaze into alignment with ($\pm 4^\circ$) either eccentric LED. The gain that could be expected from each movement was varied systematically across 5–7 blocks of about 100 trials each. A block in which each movement was reinforced equally was typically run first, and then pairs of blocks of complementary high gain/low gain conditions were randomly interleaved.

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Seeing a single photon without destroying it

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Light detection is usually a destructive process, in that detectors annihilate photons and convert them into electrical signals, making it impossible to see a single photon twice. But this limitation is not fundamental—quantum non-demolition strategies^{1–3} permit repeated measurements of physically observable quantities, yielding identical results. For example, quantum non-demolition measurements of light intensity have been demonstrated^{4–14}, suggesting possibilities for detecting weak forces and gravitational waves³. But such experiments, based on nonlinear optics, are sensitive only to macroscopic photon fluxes. The non-destructive measurement of a single photon requires an extremely strong matter–radiation coupling; this can be realized in cavity quantum electrodynamics¹⁵, where the strength of the interaction between an atom and a photon can overwhelm all dissipative couplings to the environment. Here we report a cavity quantum electrodynamics experiment in which we detect a single photon non-destructively. We use atomic interferometry to measure the phase shift in an atomic wavefunction, caused by a cycle of photon absorption and emission. Our method amounts to a restricted quantum non-demolition measurement which can be applied only to states containing one or zero photons. It may lead to quantum logic gates¹⁶ based on cavity quantum electrodynamics, and multi-atom entanglement¹⁷.

In optical quantum non-demolition (QND) measurements, a 'signal' beam is coupled to a 'meter' beam in a nonlinear medium whose refractive index depends on light intensity⁴. The phase shift of the meter resulting from the non-resonant couplings of the beams with the medium is measured by optical interferometry. The meter beam is split into two parts, one of which is coupled to the signal, the other being used as reference. The two parts are recombined before reading the meter output intensity. The signal intensity remains unaltered. In the single-photon quantum non-demolition (SP-QND) scheme described here, this procedure is modified by replacing the meter light beam with a single atom interacting resonantly with the signal field and by resorting to atomic instead of optical interferometry.

The signal field is now stored in a mode of a cavity C (Fig. 1a). Meter atoms cross C one at a time. The relevant atomic levels (e, g and i) are shown in the inset of Fig. 1a. The cavity field is resonant with the $e \Rightarrow g$ transition, while i is a reference level (the $g \Rightarrow i$ transition is non-resonant with C). Let us first assume that there is 1 photon in C and that the meter enters C in level g at time $t=0$. As the meter crosses C, the combined atom–field system undergoes a reversible Rabi oscillation¹⁸ at angular frequency Ω between the states $|g,1\rangle$ and $|e,0\rangle$ representing respectively the meter in g or e with 1 or 0 photon in C. At time t , the system is in the coherent superposition: $\cos(\Omega t/2)|g,1\rangle + \sin(\Omega t/2)|e,0\rangle$.

If the total interaction time is $2\pi/\Omega$ (2π Rabi pulse), the atom ends up in g after a full coherent cycle of photon absorption and emission and the photon number is left unchanged. The system has, however, experienced a subtle quantum alteration. The global phase of the state has changed, $|g,1\rangle$ turning into $-|g,1\rangle = e^{i\pi}|g,1\rangle$. A similar π phase shift occurs when performing a 2π rotation on a spin-1/2 particle^{19,20}. Alternatively, if C is empty, or if the atom crosses C in i, the system's state remains unaltered. As a result, a state superposition $C_g|g,1\rangle + C_i|i,1\rangle$ is transformed into

$C_g e^{i\pi}|g,1\rangle + C_i|i,1\rangle$ while, in the empty-cavity case, $C_g|g,0\rangle + C_i|i,0\rangle$ is unchanged. In short, the atomic coherence changes its phase by π if there is 1 photon in C.

The meter wavefunction phase-shift is measured by Ramsey interferometry²¹. The meter, initially in g, undergoes two interactions with a classical auxiliary field (frequency ν), before and after crossing the cavity mode (zones R₁ and R₂ in Fig. 1a). This field is nearly resonant with the $g \Rightarrow i$ transition at frequency ν_{gi} . The first pulse prepares the coherent superposition $(1/\sqrt{2})(|g\rangle + |i\rangle)$ and the second mixes the states again, probing after C the superposition phase-shift. The final atomic populations are measured downstream with a state-selective detector D. The probability of finding the meter in g or i results from a quantum interference between two paths in which the atom crosses C in either g or i. It is modulated when ν is tuned, resulting in sinusoidal 'Ramsey fringes'.

When C contains 1 photon, the amplitude associated with one of the paths (atom in g) is phase-shifted by π . The corresponding fringe pattern should thus be π out of phase with the one obtained with C empty. The interfering quantum paths and the corresponding fringes are depicted in Fig. 1b,c. Setting ν at a fringe extremum (for instance at $\nu - \nu_{gi} = 0$) results in a perfect correlation between the state of the meter (i or g) and the photon number (0 or 1).

This SP-QND scheme can measure only two photon numbers (0 and 1) in a non-demolition way. For an n -photon field with $n > 1$, the Rabi frequency is $\Omega\sqrt{n}$ and n -conservation cannot be enforced for all ns . By slightly detuning the cavity from the $e \Rightarrow g$ transition, it is however possible to suppress the meter absorption, thus avoiding completely photon demolition. The dispersive phase shift experienced by the meter in the non-resonant case is dependent upon the photon number. This shift could also be measured by Ramsey interferometry, leading now to an unrestricted QND measurement of the photon number. This dispersive method was described in our original cavity quantum electrodynamics-quantum

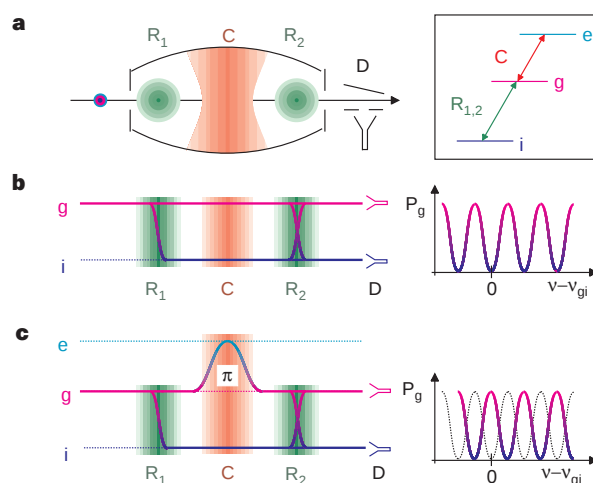


Figure 1 Scheme of our atom interferometer, which can detect single photons. **a**, Atoms cross the cavity C one at a time; the cavity mode (shown in red) stores the field to be measured. The e, g and i atomic levels are shown in the inset. The cavity mode is resonant with the $e \Rightarrow g$ transition. Atoms are subjected in zones R₁ and R₂ (in green) to auxiliary pulses (frequency ν), quasi-resonant with the $g \Rightarrow i$ transition (frequency ν_{gi}). Detector D, downstream, measures the states of the atoms. **b**, Diagram depicting the interfering paths followed by an atom initially in g when there is no photon in the cavity mode. The atom travels between R₁ and R₂ either in g (violet line) or in i (blue line). The probability amplitudes associated with these two paths interfere, leading to fringes in the probability P_g of detecting the atom in g when ν is scanned (shown at right). **c**, As **b** but when the cavity mode stores one photon. The atom, if in level g, undergoes a full reversible cycle of photon absorption and emission, finally leaving the photon in C. In the process, the corresponding amplitude has undergone a π phase shift and the fringes are reversed (as shown at right).

non-demolition proposal^{22,23}. It is, however, more difficult to implement than the resonant SP-QND scheme described here as it corresponds to a smaller phase-shift per photon for a given atom–cavity interaction time. We note that a SP-QND measurement based on a 2π Rabi pulse has already been suggested²⁴. It relied, however, on the observation of a deflection of the atomic trajectory and not on an interference effect as discussed here. Note also that multiples of 2π Rabi pulses leaving the photon number unchanged are essential to generate “trapping states”²⁵ in a micromaser.

In our set-up^{26–28}, a thermal beam of rubidium atoms is velocity-selected by laser-induced optical pumping. The atoms are then prepared in the Rydberg circular state with principal quantum number 50 (level *g*) or 51 (level *e*). The $e \Rightarrow g$ transition is resonant at 51.1 GHz. Circular Rydberg states^{29,30} have exceptionally large coupling to microwave radiation and long lifetimes (~ 30 ms for *e* and *g*), two essential features here. The circular state preparation³⁰ is pulsed. It generates at a pre-set time (within ± 1 μ s) an atomic sample with, on average, 0.3–0.6 atom. In most samples, there is zero or one atom present. This time-resolved process, combined with velocity selection, determines the atomic position at any time within ± 1 mm.

Each atom crosses the apparatus before entering a state-selective field-ionization detector D (efficiency, 30%). The interferometer operates on the 54.3-GHz $g \Rightarrow i$ transition (*i* is the circular state with principal quantum number 49). The set-up is cooled to 0.6 or 1.2 K by a helium cryostat. The average number of thermal photons in the mode at these temperatures are 0.02 and 0.15, respectively. The cavity is made of two niobium spherical mirrors in a Fabry–Perot configuration. The mirrors are surrounded by a cylindrical ring (with 3-mm holes for atom access) which reflects the photons scattered by the imperfections of the mirrors back into the cavity mode, thus reducing its losses. The resulting photon decay time is 1 ms, long enough to permit repeated field measurements.

The cavity sustains a transverse electromagnetic gaussian mode (waist $w=6$ mm), resonant with the $e \Rightarrow g$ transition. The single-photon Rabi frequency at cavity centre is $\Omega/2\pi=47$ kHz (ref. 18). The atomic velocity, $V=503(\pm 2.5)$ m s⁻¹, corresponds to an effective interaction time $t_i=\sqrt{\pi} w/V$ such that $\Omega t_i=2\pi$. A small electric field is applied across the two electrically isolated mirrors. This field is used to fine-tune the atomic frequency through the Stark effect. By commuting it at pre-set times, the $e \Rightarrow g$ transition can be put in or out of resonance with C, allowing us to reduce to a fraction of t_i the atom–cavity interaction time (see below).

The stray electric fields across the ring holes prevent atomic coherence from propagating in and out of C. We thus apply the Ramsey pulses inside the closed cavity structure, by injecting the 54.3-GHz radiation through an additional hole in the ring. This radiation does not couple with the 51.1-GHz mode. It produces a standing-wave pattern, mapped by performing time-resolved spectroscopy of the Rydberg states. This standing-wave exhibits two field antinodes sandwiching the cavity mode waist (*R*₁ and *R*₂ in Fig. 1a). As we know the position of each atom as a function of time, we can apply controlled pulses when the meter crosses *R*₁ and *R*₂. The resulting fringes, detected by accumulating statistics of atom counts in *g* and *i*, have a 72% contrast (*g* and *i* states probabilities oscillating between 0.86 and 0.14).

An experimental sequence consists of preparing a small field in C, then measuring it by sending a meter atom across the interferometer, possibly checking the result with another atom. Statistics are accumulated over an ensemble of sequences. Since each atom sample has a 0.1–0.2 probability of yielding a signal, the probability of detecting an atom pair is only 1–4%. To limit data acquisition time, sequences are repeated after 1.5 ms, without waiting for complete cavity relaxation. A photon erasing procedure is used to remove residual photons at the start of each sequence. Photon erasing is also important because the waveguide injecting the

Ramsey pulses leaks into C a small thermal field, corresponding to an average of 0.7 photons (this value is deduced from a measurement of the $g \Rightarrow e$ absorption rate of an atom crossing C). Each sequence starts by sending five pulses of atoms prepared in *g*. These eraser pulses contain three to nine atoms each. They absorb the remaining field, cooling the mode down to 0.12 photons on average. The experimental sequence is then performed within 400 μ s, to limit thermal field build-up.

In a first experiment, we use a source atom to generate a single photon²⁸. This atom, prepared in *e*, is sent across C, 100 μ s after the last eraser pulse. The interaction time of the source with the mode is reduced to $t_i/4$ by Stark-tuning the $e \Rightarrow g$ transition while the atom is in C. The atom–field system undergoes a $\pi/2$ Rabi pulse and is prepared in the entangled state $(1/\sqrt{2})(|e,0\rangle+|g,1\rangle)$. Detecting the source in *e* (*g*) ideally reduces the field to a 0 (1)-photon state. We thus prepare, with equal probabilities, either 0 or 1 photon.

We then send the meter after a 100- μ s delay. The interaction time is reset to the value t_i . The 2π Rabi pulse is slightly imperfect, with a 20% residual probability for the meter to absorb the photon. The conditional probabilities $P_{g2/e1}(\nu)$ and $P_{g2/g1}(\nu)$ to detect the meter in *g*, provided the source has been detected in *e* or *g* respectively, are reconstructed as a function of the Ramsey frequency ν . About 250 events are sampled per frequency step. Ideally, these probabilities are equal to the conditional probabilities $P_{g/0}(\nu)$ and $P_{g/1}(\nu)$ of detecting the meter in *g* if there is 0 or 1 photon in C.

As expected, we obtain two sinusoidal fringe patterns, π phase-shifted with respect to each other (Fig. 2). The fringes corresponding to the source detected in *e* have the same phase as the ones obtained with C empty. Their contrast is limited to 41% by various known imperfections (see Fig. 2 legend). The lines in the figure result from a simulation taking these imperfections into account. The agreement with the experiment is very good, meaning that the limitations of the apparatus are well understood.

The CQED interferometer is characterized by the sinusoidally varying conditional probabilities $P_{a/0}(\nu)$ and $P_{a/1}(\nu)$, where *a* stands for *g* or *i*. In a practical measurement, the photon number is initially unknown and we are interested in the reverse conditional probabilities

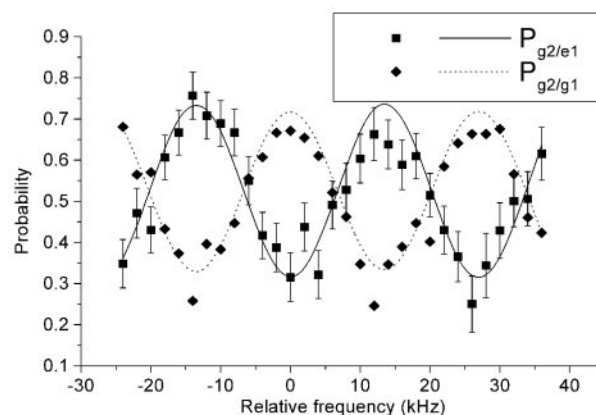


Figure 2 Preparing and detecting one photon. A first atom prepares 0 or 1 photon and a second atom performs an interferometric measurement: recordings of the conditional probabilities $P_{g2/e1}(\nu)$ (squares) and $P_{g2/g1}(\nu)$ (diamonds) of detecting the second atom in *g* provided the first has been found in *e* or *g* (leaving 0 or 1 photon in C). These probabilities are reconstructed as a function of ν by averaging 250 correlated atom counts for each ν value (experimental points; statistical error bars are shown only for $P_{g2/e1}(\nu)$ for the sake of clarity). The two signals, π phase shifted with respect to each other, demonstrate the operation of the single photon sensitive interferometer. The lines result from numerical simulation considering known apparatus imperfections: 72% contrast of the Ramsey interferometer, imperfections of the $\pi/2$ and 2π Rabi pulses, detection errors, samples containing two atoms, residual thermal field in C, and field relaxation between source and meter.

$P_{1/a}(\nu)$ and $P_{0/a}(\nu)$ of having 0 or 1 photon in C after the meter has been found in a given state. A simple application of Bayes laws yields:

$$P_{1/a}(\nu) = P_1 \cdot P_{a/1}(\nu) / [P_1 \cdot P_{a/1}(\nu) + P_0 \cdot P_{a/0}(\nu)] \quad (1)$$

where P_0 and P_1 are the probabilities of having, before measurement, respectively 0 and 1 photon in C (other photon numbers are outside the range of the SP-QND scheme). Equation (1) describes how the information acquired by the meter reading changes the probability of having 1 photon.

To check the physical content of equation (1), we perform a measurement on a small thermal field (average photon number: $0.30(\pm 0.02)$) which builds up in C during a 300- μ s delay between the last eraser pulse and the meter. The *a priori* photon number probabilities are $P_0 = 0.77$ and $P_1 = 0.18$, with a negligible probability (0.05) of having more than one photon. Following the meter, we send an absorbing probe atom across C. The probe is prepared in g, 75 μ s after the meter, and does not undergo any Ramsey pulse. Its interaction time with C, $t_i/2$, corresponds to a π Rabi pulse in 1 photon. The probability of detecting the probe in e is hence ideally equal to the probability of finding 1 photon in C after the SP-QND measurement.

Figure 3 shows the measured conditional probabilities $P_{e2/g1}(\nu)$ (squares) and $P_{e2/i1}(\nu)$ (diamonds) of detecting the probe in e, provided the meter is detected in g or i, as a function of ν . We have also recorded the probability $P_{e2}(\nu)$ of detecting the probe in e, when no meter is sent across C (triangles). The lines in Fig. 3, obtained by a simulation taking into account the imperfections listed in Fig. 2 legend, are in very good agreement with the experiment.

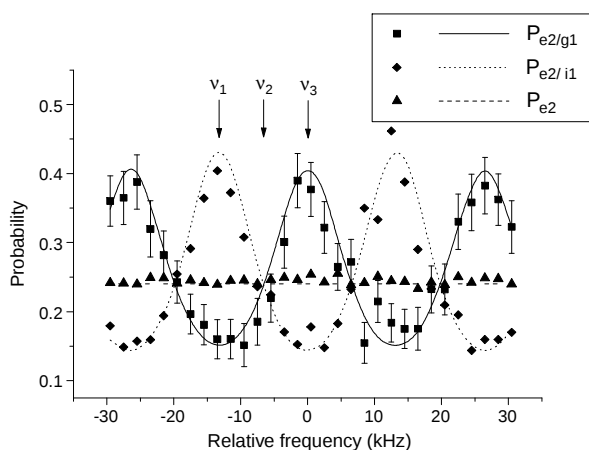


Figure 3 Measuring a photon twice. A first meter atom performs a QND measurement of a small thermal field, and a second probe atom absorbs the field left afterwards. Squares and diamonds indicate experimental points showing the conditional probabilities $P_{e2/g1}(\nu)$ (squares) and $P_{e2/i1}(\nu)$ (diamonds) of finding the probe in e (having absorbed a photon in C), provided that the meter has been measured in g or i. About 650 atom pairs are averaged per frequency step. The probabilities are reconstructed as a function of ν , and the lines are obtained by numerical simulations. The triangles correspond to the probability of finding the probe in e when no measurement is performed on the meter. The periodic variations of the conditional signals show how the information acquired about the field by the first measurement modifies the probability of finding one photon in C. The effect of this measurement depends upon the frequency setting of the interferometer. Three particular frequencies ν_1 , ν_2 and ν_3 are indicated by the arrows. At ν_1 , 1 photon in C results in a maximum (minimum) probability of finding the meter in i (g). If it is found in i, it is likely that there is 1 photon left in C afterwards. The *a posteriori* probability of finding 1 photon is then larger than the *a priori* probability P_1 . The probability that the probe is excited in e exceeds P_{e2} (line of triangles). If the meter is found in g, there is conversely a large probability that C is empty afterwards and the probe excitation probability falls below P_{e2} . At ν_2 , the meter indication is ambiguous and the *a posteriori* probe excitation probabilities, equal for both meter states, correspond to the *a priori* probability. At ν_3 , level g indicates 1 photon, and the conclusions are opposite to those for ν_1 .

The line of triangles represents the average atomic absorption rate of the initial field. The difference between the observed value of P_{e2} , 0.24, and $P_1 = 0.18$ is due to apparatus imperfections. The lines of squares and diamonds show that the probability of having 1 photon in C after reading the meter is either smaller or larger than P_1 , depending upon the outcome of the measurement and upon ν (see Fig. 3 legend). The non-demolition feature of the SP-QND measurement is clearly revealed here. When the meter finds 1 photon, it does so without destroying it, leaving it with a high probability behind to excite the probe atom. The detection process by this probe is quite different, as it leaves C empty at the end. The difference between the normal (absorptive) and SP-QND measuring schemes appears clearly here.

We note also that the meter, injected in the lower state of the transition resonant with C, cannot add any energy to the mode. However, detecting it in the state correlated to 1 photon increases the probability of the probe absorbing energy. This might seem paradoxical. In fact, our information about the field (initially described by P_1) is modified by the meter reading (see equation (1)). The change in the probe's absorption rate reflects the fact that we have gained information on the field. Averaging this absorption rate weighted by the respective probabilities of finding the meter in e and g amounts to neglecting the meter information. This average recovers the value corresponding to P_1 .

We have also performed a repeated SP-QND measurement, sending two identical meter atoms, 75 μ s apart, to detect twice a thermal field with $P_1 = 0.18$. The interferometer was set at frequencies ν_1 and ν_3 (defined in Fig. 2), and, in each case, 1,000 coincidence events were recorded. The conditional probability of finding the second meter in the same state as the first is larger than the *a priori* probability. For example, at ν_1 , the *a priori* probability of finding the atom in i is $0.32(\pm 0.01)$, whereas the conditional probability $P_{i2/i1}$ is increased to the value $0.48(\pm 0.02)$. Ideally, these figures should be equal to 0.18 and 1. The observed values are correctly predicted by our numerical model.

We have achieved a single-photon-QND measurement satisfying the criteria of non-demolition and repeatability. Applying our analysis of the interferometer imperfections to the detection of an unknown field containing no more than one photon ($P_0 = P_1 = 0.5$), we infer that, after a single meter reading, the corresponding photon number probability increases to 0.84. Other developments are in progress. The SP-QND process entangles the 0-1 photon field and the meter. The combined system evolves, before atomic detection, into a coherent superposition of correlated atom-photon states, according to a conditional dynamics in which the photon state controls the atomic state. The SP-QND scheme can be described in terms of quantum logic, and amounts to the operation of a quantum gate¹⁶ in which the photon and the atom play respectively the roles of control and target qubits. By performing a SP-QND measurement of a small coherent field, we are at present studying the coherent operation of this gate. Finally, by allowing us to entangle in a deterministic way several atoms crossing C one at a time, the SP-QND process provides a building block for a general method to entangle an arbitrary number of atoms. A demonstration of three-atom entanglement is under way. □

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Synthesis of nuclei of the superheavy element 114 in reactions induced by ^{48}Ca

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The stability of heavy nuclides, which tend to decay by α -emission and spontaneous fission, is determined by the structural properties of nuclear matter. Nuclear binding energies and lifetimes increase markedly in the vicinity of closed shells of neutrons or protons (nucleons), corresponding to ‘magic’ numbers of nucleons; these give rise to the most stable (spherical) nuclear shapes in the ground state. For example, with a proton number of

$Z = 82$ and a neutron number of $N = 126$, the nucleus ^{208}Pb is ‘doubly-magic’ and also exceptionally stable. The next closed neutron shell is expected at $N = 184$, leading to the prediction of an ‘island of stability’ of superheavy nuclei, for a broad range of isotopes with $Z = 104$ to 120 (refs 1, 2). The heaviest known nuclei have lifetimes of less than a millisecond, but nuclei near the top of the island of stability are predicted to exist for many years. (In contrast, nuclear matter consisting of about 300 nucleons with no shell structure would undergo fission within about 10^{-20} seconds.) Calculations^{3–5} indicate that nuclei with $N > 168$ should already benefit from the stabilizing influence of the closed shell at $N = 184$. Here we report the synthesis of an isotope containing 114 protons and 173 neutrons, through fusion of intense beams of ^{48}Ca ions with ^{242}Pu targets. The isotope decays by α -emission with a half-life of about five seconds, providing experimental confirmation of the island of stability.

Neutron-rich nuclei with $N > 168$ can be synthesized, as we have shown earlier⁶, in fusion reactions using the heaviest isotopes of uranium, plutonium and curium as targets and a ^{48}Ca ion beam. As a result of the significant mass defect of the doubly magic ^{48}Ca nucleus (magic numbers $N = 28$ and $Z = 20$), the excitation energy (E_x) of the compound nucleus at the Coulomb barrier only amounts to about 30 MeV. This corresponds to an energy $E_{\text{lab}} = 230$ –235 MeV of the ^{48}Ca bombarding projectiles. The de-excitation of this nucleus should proceed mainly by the emission of three neutrons and γ -rays^{7,8}. Calculations predict that the probability for the excited compound nucleus to reach the final state (evaporation residue, EVR) after the evaporation of 2 or 4 neutrons is one order of magnitude less at the bombarding ion energy near the Coulomb barrier. This circumstance should increase the survival probability of the EVRs as compared with the case of hot fusion reactions ($E_x \approx 50$ MeV), which were used for the synthesis of heavy isotopes of elements with atomic numbers $Z = 106$, 108 and 110 (refs 9–11). On the other hand, the high asymmetry of the interacting nuclei in the entrance channel ($A_p/A_T = 0.2$, $Z_p/Z_T = 1.880$, where A_p , A_T and Z_p , Z_T are mass and atomic numbers of the projectile and target nuclei respectively) should decrease possible dynamical limitations¹² on the fusion of massive nuclei as compared with more symmetrical cold fusion reactions.

In spite of these obvious advantages, previous attempts to synthesize new elements in ^{48}Ca -induced reactions gave only the upper limits of the production cross-sections of superheavy elements^{13–15}. As is apparent now, this could be explained by the low experiment sensitivity, as the intensity of the ^{48}Ca beam was not high enough.

The first positive result was obtained in spring 1998 in the $^{48}\text{Ca} + ^{238}\text{U}$ reaction with a total beam dose of 3.5×10^{18} Ca ions. Two spontaneous fission events were observed, which were assigned to the decay of a new isotope of element 112 produced in the reaction $^{238}\text{U}(^{48}\text{Ca}, 3n)^{283}112$ with a cross-section of $\sigma_{3n} = 5^{+6}_{-3}$ picobarn (pb, 10^{-36} cm^2)¹⁶. The cross-section value of 1 pb corresponds to the observation of one wanted event within 4 days, providing that the beam intensity is 4×10^{12} particles per second, the number of target nuclei able to take part in the fusion reaction is 5×10^{17} and the experiment efficiency is 100%. The half-life of the new nuclide against spontaneous fission (T_{SF}), determined on the basis of the two events, was ~ 1.5 min. This is about 3×10^5 times longer than the α -decay half-life (T_{α}) of the known lighter isotope of element 112, synthesized in the reaction $^{208}\text{Pb}(^{70}\text{Zn}, \text{In})^{277}112$ by Hofmann *et al.*¹⁷ in 1996 (Fig. 1a).

The next experiment, performed at the end of 1998, was aimed at the synthesis of nuclei with $Z = 114$ in the reaction $^{48}\text{Ca} + ^{244}\text{Pu}$. In a 34-day irradiation with a beam dose of Ca ions of 5.3×10^{18} , a decay chain—consisting of three sequential α -decays and spontaneous fission, taking about 34 min in all—was observed after the implantation of a heavy atom in the detector¹⁸. This decay chain may be considered as a good candidate for originating from

the α -decay of the parent nucleus $^{289}_{114}$ ($T_{\alpha} \approx 20$ s), produced in the 3n-evaporation channel with a cross-section of ~ 1 pb. It is very important to have at least two isotopes of element 114 for establishing a certain trend in the half-lives—whether they increase with an increase in the neutron number, thereby indicating that we are reaching the shore of ‘the island of stability’.

If the identification performed in the above two experiments is correct, then it is not difficult to predict the properties of the new isotope of element 114. According to the calculations and the above mentioned data, the isotope $^{287}_{114}$ ($N = 173$) should undergo predominantly α -decay to the daughter nucleus $^{283}_{112}$, obtained earlier in the $^{48}\text{Ca} + ^{238}\text{U}$ reaction. Thus one could expect a short

decay chain, α -SF, with a relatively short half-life with respect to α -decay (of several seconds) and a subsequent spontaneous fission (SF) with a considerably longer half-life (of several minutes). Here we report another experiment also aimed at the synthesis of nuclei with $Z = 114$ in the reaction $^{48}\text{Ca} + ^{242}\text{Pu}$.

The expected cross-section for the production of the new isotope $^{287}_{114}$ at the maximum of the excitation function for the 3n-evaporation channel is very small (~ 1 pb; refs 7, 8). This requires high sensitivity in the experiment. From this point of view, the production of an intense ion beam of the rare and expensive isotope ^{48}Ca is the cornerstone of our attempts to synthesize superheavy elements.

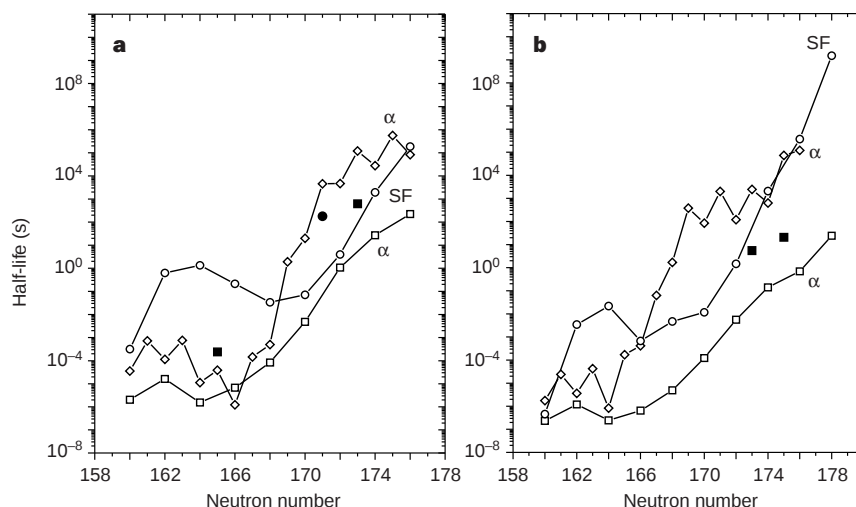


Figure 1 Theoretical predictions for partial half-lives of isotopes. **a**, With $Z = 112$. Open circles and open squares connected by solid lines are respectively T_{SF} and T_{α} (from ref. 4). Open diamonds connected by solid lines are T_{α} (from ref. 5). Filled square for $N = 165$, experimental T_{α} for the isotope $^{277}_{112}$ from ref. 17; filled square for $N = 173$, experimental T_{α} for the isotope $^{285}_{112}$ from ref. 18; filled circle for

$N = 171$, experimental T_{SF} for the isotope $^{283}_{112}$ from ref. 16 and this work. **b**, With $Z = 114$. Open circles and open squares connected by solid lines are respectively T_{SF} and T_{α} from ref. 4; open diamonds connected by solid lines are T_{α} taken from ref. 5; filled square for $N = 173$, experimental T_{α} for the isotope $^{287}_{114}$ from this work; filled square for $N = 175$, experimental T_{α} for the isotope $^{289}_{114}$ from ref. 18.

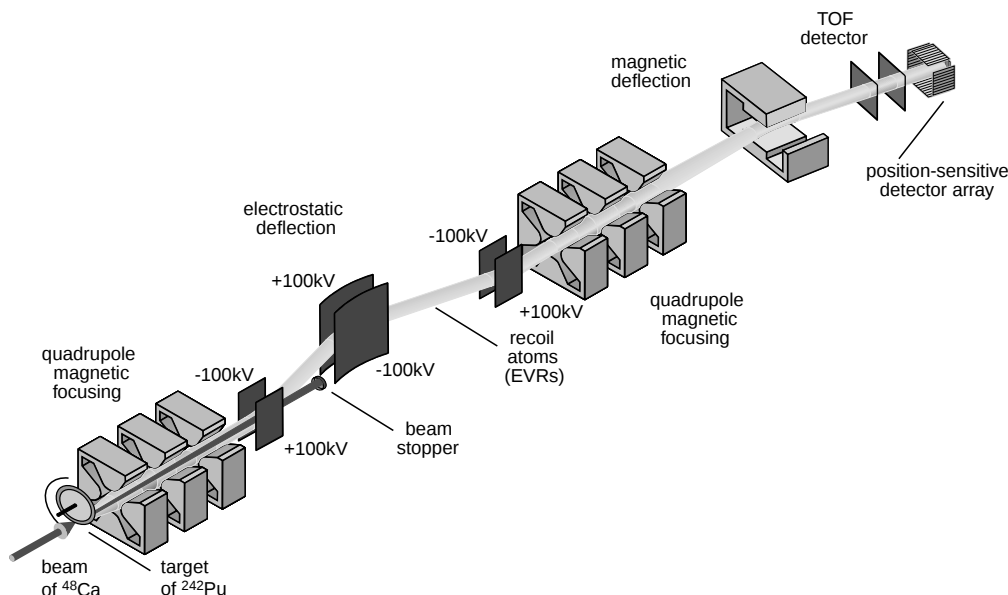


Figure 2 The electrostatic recoil separator VASSILISSA. The main parameters of the separator (the values of the electric fields of the electrostatic deflectors, and the values of the magnetic fields of the quadrupole lenses and the dipole magnet) and the transmission value of the EVR from the reaction $^{48}\text{Ca} + ^{242}\text{Pu}$ were estimated from test experiments. The cross-sections of various xn-evaporation channels were measured in irradiations of ^{159}Tb , ^{174}Yb and $^{206,208}\text{Pb}$ targets with ^{48}Ca projectiles of different energies. In these reactions, the well known At, Th and

No isotopes undergoing α -decay and spontaneous fission (^{252}No) were detected. According to the data from the test reactions, the EVR of element 114 will have a mean energy of 36 MeV and a corresponding mean ionic charge state (q) = 18. Under these conditions $\sim 30\%$ of the $^{290-x}_{114}$ EVR, produced with a ^{242}Pu target, would be implanted into the detector located at the focal plane of the separator at a distance of 12 m from the target.

The low-energy ($E_{\text{lab}} = 60 \text{ keV}$) $^{48}\text{Ca}^{5+}$ beam was extracted from the ECR ion source¹⁹ and injected into the Dubna U400 heavy-ion cyclotron, where it was accelerated; the beam was then extracted from the cyclotron and transported to the experimental target. The average intensity of the ion beam on the target was 4×10^{12} particles per second at a material consumption rate of $\sim 0.3 \text{ mg h}^{-1}$. The beam energy was determined with a precision of $\sim 1 \text{ MeV}$ by measuring the energies of the scattered ions and using a time-of-flight (TOF) technique.

The targets consisted of the enriched isotope ^{242}Pu (97%) in the form of PuO_2 , deposited onto 1.7- μm -thick Ti foils with a surface density of 0.2 mg cm^{-2} . Each target had an area of 5 cm^2 in the shape of an arc segment with an angular extension of 60° and an average radius of 55 mm. Six targets were mounted on a disk that was rotated at 2,000 r.p.m. perpendicularly to the beam direction. A 'chopper' system switched out the beam to make a pause (0.6 ms) at the moment when the target disk frames passed the beam. The EVRs were separated in-flight from the beam particles and other reaction products by the VASSILISSA²⁰ electrostatic recoil separator (Fig. 2).

For the registration of the EVRs and their radioactive decay, a system of TOF detectors and a silicon position-sensitive strip-detector array was installed in the focal plane of the separator. After registration by the TOF detectors—consisting of two secondary-electron foil converters with microchannel plates—the recoil atoms were implanted into a 16-strip silicon detector, which had an active area of $60 \times 60 \text{ mm}^2$. Each strip had a longitudinal position sensitivity. A 3- μm -thick degrader foil (Mylar) was placed in front of the silicon detectors to reduce the number of low-energy projectiles reaching the focal plane of the separator. Measurements of the position resolution along each strip were performed. For sequential $[\alpha-\alpha]$ decays it was 0.6 mm (full-width at half-maximum, FWHM), for correlated [EVR- α] it was 1 mm, and for [EVR-SF] it was $\sim 1.5 \text{ mm}$. The measurements were performed for recoil nuclei with energies of 4 to 15 MeV, which fully covered the expected energy interval for the signals originating from the implanted $Z = 114$ nuclei.

In order to increase the detection efficiency for α -particles, the front detector was surrounded by four silicon detectors of the same size as the stop detector. The entire array had the shape of a cube with a 60-mm edge length. The efficiency of the silicon array for the detection of α -particles with the full energy was 85% of 4π . Thus, the probability that the α -particle energy (E_α) was determined

directly by the front detector was 50% (energy resolution $\Delta E_\alpha = 20 \text{ keV}$); it was 35% when E_α was the sum of two signals $E_\alpha = E_{\alpha 1} + E_{\alpha 2}$ from the front and side detectors (energy resolution $\Delta E_\alpha = 150 \text{ keV}$); and finally, it was 15% if the α -particle were emitted backwards leaving only an escape signal ($E_{\alpha 1}$) in the front detector if $E_{\alpha 1} \geq 1 \text{ MeV}$. In recording the events, the accuracy of the time registration was $\sim 1 \mu\text{s}$.

The signals from the TOF detectors were used to measure the velocity of the recoils and to distinguish the radioactive decays of previously implanted nuclei. The high efficiency of these detectors made it possible to obtain very clean decay spectra. The time window for measuring decay chains could be considerably widened (up to several hours). The latter was particularly important for the correlation of decays with long half-lives in the presence of a continuously running beam.

At a beam intensity of $\sim 4 \times 10^{12} \text{ ions s}^{-1}$, the total counting rate of all events at the focal plane detector was only 25–30 events s^{-1} . The counting rate at a single strip in a position interval of 1.2 mm amounted to the following values; for the α -like signals (in the absence of signals from the TOF detector) with an energy higher than 7.5 MeV, less than 1 h^{-1} ; for the α -like signals with an energy of 1–4 MeV (signals corresponding to escaped α -particles), about 3 h^{-1} ; for recoil-like (EVR-like) signals (with a TOF signal) with an energy higher than 4 MeV, about 4 h^{-1} .

The experiment was performed during 3 March to 5 April 1999. The beam energy in the middle of target was $E_{\text{lab}} = 235 \pm 2 \text{ MeV}$. Over a period of 32 days, a total of 7.5×10^{18} Ca projectiles was passed through the target. In the analysis of the experimental data, we proceeded from the expected short decay chain of $^{287}114$ terminated by spontaneous fission of the daughter nucleus (or its grand-daughters). So, first of all, the decay events with a large energy deposit in the front detector ($E > 100 \text{ MeV}$) were selected. Four such events were observed in the reaction $^{48}\text{Ca} + ^{242}\text{Pu}$.

Two events with energies $E = 144 \text{ MeV}$ and 175 MeV were registered respectively 59 μs and 20 μs after the implantation of the corresponding position-correlated recoil atoms. We assigned these events to the SF-isomers, tentatively to the 24- μs $^{241\text{m}}\text{Pu}$, produced in a transfer reaction, and being a neighbour of the ^{242}Pu -target nucleus. For the other two events, spontaneous fission was observed as two coincident signals (two fission fragments) with an energy $E_{\text{F1}} = 130 \text{ MeV}$ deposited in the front detector and $E_{\text{F2}} = 65 \text{ MeV}$ in the side detector, with $E_{\text{tot}} = 195 \text{ MeV}$ for the

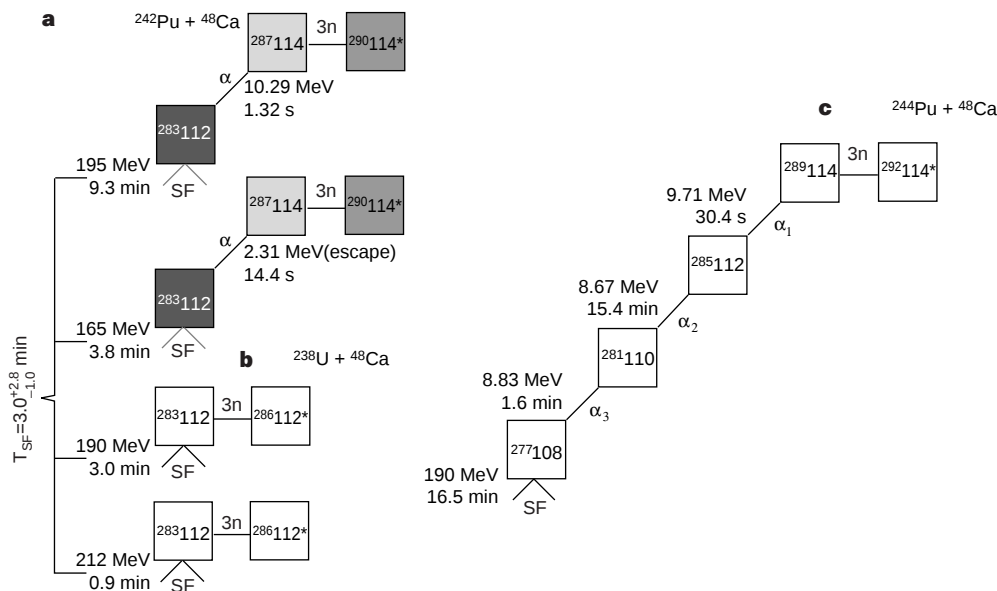


Figure 3 Position-correlated decay chains. **a**, Decay chain of $^{287}114$, produced in the reaction $^{48}\text{Ca} + ^{242}\text{Pu}$ (present work); **b**, of $^{283}112$, produced in the reaction

$^{48}\text{Ca} + ^{238}\text{U}$ (ref. 16); and **c**, of $^{289}114$, produced in the reaction $^{48}\text{Ca} + ^{244}\text{Pu}$ (ref. 18).

first event; and $E_{F1} = 110$ MeV, $E_{F2} = 55$ MeV, with $E_{\text{tot}} = 165$ MeV for the second event.

We searched the recorded data backwards in time from each of those two events for preceding α -particles correlated in position. For the first case only one α -particle was detected in the front detector 1.32 s after the implantation in the middle of the strip of a recoil nucleus with a measured energy $E_{\text{EVR}} = 10$ MeV. This value agrees with the energy expected for the element-114 recoils, and the TOF signal is consistent with that expected for a complete-fusion EVR, as determined in the calibration reactions. The energy of the α -particle was $E_{\alpha} = 10.29$ MeV. The SF event was observed 559.6 s later. All the three signals (EVR, α and SF) appeared within a position interval of 0.82 mm, which indicates that there is correlation between the observed decays. The probability for the observed correlation to be a random coincidence of signals imitating the decay chain (EVR, α and SF) at a given position window amounts to $\sim 2 \times 10^{-4}$. It was calculated employing a method used in the case of low statistics²¹.

In the second case, spontaneous fission was observed 243 s after the registration of the implanted recoil nucleus with the energy $E_{\text{EVR}} = 13.5$ MeV. In the search for an α -particle in the time interval EVR–SF, only a single escape signal with $E_{\alpha 1} = 2.31$ MeV was found 14.45 s after the implantation of the recoil nucleus. All the three signals (EVR, α and SF) appeared within a position interval of 1.0 mm, which again indicated correlation between the observed decays. The probability for the observed correlation to be a random coincidence of signals of the EVR, α and SF type in this case is 3×10^{-3} . The entire position-correlated decay chains are shown in Fig. 3a. In both sequences, the parent nucleus undergoes α -decay. As the total energy E_{α} for the second event is not determined, we assume that the α -decay in both the cases proceeds from one and the same state. The decay properties of the parent nucleus are $T_{\alpha} = 5.5^{+10}_{-2}$ s and $E_{\alpha} = 10.29 \pm 0.02$ MeV (determined by one event).

In the experiment described here, namely $^{48}\text{Ca} + ^{242}\text{Pu}$ reaction, 2 neutrons and 2 protons were added to the target in comparison with the previous experiment $^{48}\text{Ca} + ^{238}\text{U}$. In this case after the α -decay (2 neutrons and 2 protons) the EVR of 114 will be transformed to the daughter nucleus with $Z = 112$. It is most likely that the $^{283}112$ daughter nuclei from the reaction $^{48}\text{Ca} + ^{242}\text{Pu}$ are the same as the nuclei produced directly in the reaction $^{48}\text{Ca} + ^{238}\text{U}$ (ref. 16) and all of them undergo spontaneous fission. The reason is that the bombarding energy of ^{48}Ca ions and the corresponding excitation energy of compound nuclei $^{286}112$ and $^{290}114$ are very close (in their values) and these compound nuclei could evaporate the same number of neutrons (most probably 3 neutrons). The time intervals measured for all the four spontaneous fission events (this work and ref. 16), as can be seen from Fig. 3a and b, correspond to the same half-life within the limits of the probability errors. It is then possible to conclude that in both the reactions we observe the spontaneous fission of one and the same nuclide with $T_{\text{SF}} \approx 3$ min. In the $^{48}\text{Ca} + ^{238}\text{U}$ reaction it was produced directly as an EVR in the 3n-evaporation channel, while in the $^{48}\text{Ca} + ^{242}\text{Pu}$ reaction it is the daughter from the α -decay of the parent $^{287}114$ nucleus. The half-life of the nucleus $^{283}112$ determined on the basis of the four events amounts to $T_{\text{SF}} = 180^{+170}_{-60}$ s. From the analysis of the data, we conclude that the observed α -SF chains arise with a high probability from the decay of the superheavy nucleus, which is the isotope of element 114 with the mass number 287 ($Q_{\alpha} = 10.44 \pm 0.02$ MeV, where Q_{α} is the total energy deposited at α -decay). That nucleus could be produced in the 3n-evaporation channel with the cross-section $\sigma = 2.5^{+3.3}_{-1.6}$ pb.

The half-life of the new isotope $^{287}114$ is several times shorter than that of the previously observed heavier isotope $^{289}114$, formed in the reaction $^{48}\text{Ca} + ^{244}\text{Pu}$ (Fig. 3c). Such a trend is expected with a decrease in the neutron numbers of the superheavy nuclei (Fig. 1b).

The observed radioactive properties of the new nucleus $^{287}114$, together with the data obtained earlier for the isotope $^{289}114$ and the products of its α -decay (namely, the isotopes $^{283}112$ and $^{285}112$; refs 16, 18) can be considered as experimental proof of the existence of 'the island of stability' of superheavy elements. □

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Observation of negative electron-binding energy in a molecule

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In neutral atoms and molecules, electrons are kept within their orbitals by attractive electrostatic interactions with positively charged nuclei, with relatively few neutral molecules being able to bind more than one extra electron. For multiply charged molecular anions, dynamic stability plays an important role: the superposition of long-range Coulomb repulsion and short-range electron binding gives rise to a repulsive Coulomb barrier (RCB) that traps the excess electrons¹. The RCB has profound effects on the physical and chemical properties of multiply charged anions

in the gas phase^{1–5}. For example, it has recently been shown to prevent the detachment of electrons from a doubly charged anion, even when the excitation energies exceed the electron binding energy^{6,7}. Here we report photodetachment experiments which demonstrate that the RCB can even trap electrons in molecular orbitals characterized by a negative binding energy. We show that the addition of sulphonate groups ($-\text{SO}_3^-$) to cyclic copper phthalocyanine (CuPc; ref. 8) systematically increases the energy of the corresponding molecular orbitals, culminating in the highest occupied molecular orbital of the tetra-anion, $[\text{CuPc}(\text{SO}_3)_4]^{4-}$, being unstable by 0.9 eV. The increase in molecular orbital energy and the negative electron binding energy we observe are due to charge localization in the sulphonate groups and the resultant RCB. The unusually large height of the repulsive barrier also ensures that the anion remains metastable, and continues to store 0.9 eV excess electrostatic energy, throughout the 400 seconds we are able to observe it.

Details of the experimental apparatus have been given elsewhere⁹. Our experiment involves generation of gas-phase multiply charged negative ions using electrospray and photodetachment photoelectron spectroscopy (PES) of size- and charge-selected anions. The electrospray solution is a 10^{-4} M CuPc-3, 4', 4'', 4'''-tetrasulphonate sodium salt solution in a water/methanol (2/98 ratio) mixed solvent at neutral pH. The anions produced from the electrospray are accumulated and stored in a quadrupole ion trap for 0.1 s at a pressure of 10^{-4} torr before being analysed by a time-of-flight mass spectrometer. We use two detachment photon energies ($h\nu$), 6.424 eV (193 nm wavelength) and 4.661 eV (266 nm), and measure the kinetic energies (KE) of the photoemitted electrons using a magnetic-bottle time-of-flight electron analyser. The measured distributions of electron times of flight are converted to KE spectra calibrated using the known spectra of I^- and O^- . The electron binding energies (BE) are determined from Einstein's photoelectric equation, $h\nu = \text{BE} + \text{KE}$.

Figure 1a and b shows the spectra of $[\text{CuPc}(\text{SO}_3)_4]^{4-}$ tetra-anions at the two detachment energies. The 193-nm spectrum reveals a weak feature (X) at negative binding energies with a threshold energy of -0.9 eV and two broad features (A and B) at positive binding energies. The observation of the negative-binding-energy feature is remarkable, indicating that the tetra-anion is unstable with respect to an electron loss. The photoelectron KE corresponding to the negative-binding-energy feature is 7.32 eV at 193 nm, that

is, 0.9 eV higher than the photon energy. The electron energy scales are carefully calibrated and the surprising observation of the negative electron binding energy is reproducible (see also Fig. 1 legend). At 266 nm, the negative-binding-energy feature becomes dominant; the higher-binding-energy feature B disappears and only a tail of feature A is observed. Figure 1c and d shows the spectra of the triply charged monoprotanated species, $[\text{CuPc}(\text{SO}_3)_4\text{H}]^{3-}$, at 266 and 193 nm. The features of the 193-nm spectrum of the trianion (Fig. 1d) is similar to that of the tetra-anion (Fig. 1b) except that the trianion spectrum is shifted to higher binding energies with a threshold energy at 1.2 eV. Again at 266 nm, the higher-binding-energy features of the trianion spectrum disappear (Fig. 1c). The photon-energy dependence of the PES features of both the tetra- and trianions are consistent with the existence of the RCB and the multiply charged nature of these species^{6,7}.

We note that the PES spectra of Fig. 1b and d are rather similar to that of the neutral parent CuPc molecule in the vapour phase except that neutral CuPc has a much higher BE with a threshold ionization potential of 6.3 eV (ref. 10). The similarity between the PES spectra of the present charged species and that of the parent CuPc suggests that detachment occurs from molecular orbitals of mainly CuPc character in the tetra- and trianions. This can be understood from the localized nature of the $-\text{SO}_3^-$ groups and the electrostatic interactions within the charged species. Our PES spectra of the benzene-sulphonate anion ($\text{C}_6\text{H}_5-\text{SO}_3^-$) and other singly charged sulphonate species show two detachment features from the $-\text{SO}_3^-$ group separated by 0.6 eV with a threshold binding energy of ~ 5 eV (X.-B.W. and L.-S.W., unpublished results). In the tetra-anion, the electron binding energy in $-\text{SO}_3^-$ is expected to be reduced owing to the Coulomb repulsion from the other three negative charges. Figure 2 shows a schematic molecular structure of the tetra-anion and the estimated distances between the charge centres and between the central Cu atom and the charge centres¹¹. The $-\text{SO}_3^-$ group at position 1 experiences the largest Coulomb repulsion, which amounts to ~ 3.8 eV ($\Sigma_{n=2}^4 e^2/R_{1n}$). Therefore, electrons on this $-\text{SO}_3^-$ group are still expected to be bound by at least 1.2 eV ($5 - 3.8$ eV). However, the four negative charges create a 7.2 eV ($\Sigma_{n=1}^4 e^2/R_{n5}$) negative potential on the central Cu atom; that is, an electron localized on Cu would experience a Coulomb repulsion of 7.2 eV. Previous density functional calculations predict that the highest occupied molecular orbital (HOMO) of CuPc is a Cu d_π orbital with a single occupancy ($11b_{2g}$, Fig. 3a)¹².

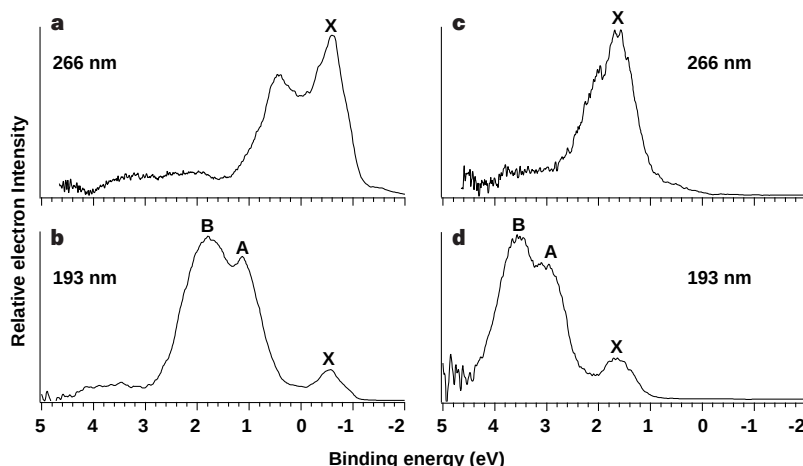


Figure 1 Photodetachment spectra of $[\text{CuPc}(\text{SO}_3)_4]^{4-}$ and $[\text{CuPc}(\text{SO}_3)_4\text{H}]^{3-}$ at 266 and 193 nm. **a** and **b**, $[\text{CuPc}(\text{SO}_3)_4]^{4-}$; **c** and **d**, $[\text{CuPc}(\text{SO}_3)_4\text{H}]^{3-}$. Note the negative electron-binding-energy feature (X) of the tetra-anion and the rigid shift to higher binding energies of the trianion spectra. The energy scale is calibrated before and after each experiment to ensure accurate measurements of electron kinetic energies. The energy shifts are typically <10 meV within a 14-h experiment. The spectra are measured several times a month and are highly reproducible, being

independent of source conditions (temperature and pressure). Multiphoton processes are ruled out because the same negative-binding-energy feature is observed with different photon fluxes and at various detachment laser wavelengths. Population of an excited state of the tetra-anion may give a negative electron-binding energy, but is unlikely owing to the multiple-collision conditions of the ion source and ion-trap, and the relatively long timescale of the experiments.

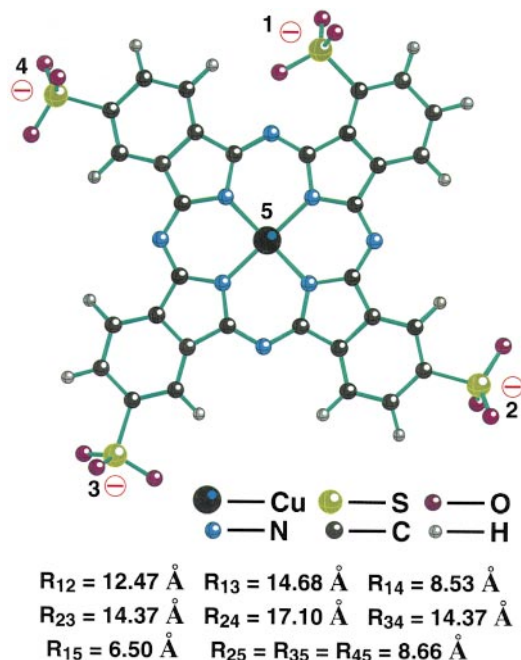


Figure 2 Structure of the $[\text{CuPc}(\text{SO}_3)_4]^{4-}$ tetra-anion as determined in bulk crystals (ref. 11). The location of the charges, and the distances between the charge centres and between the charge centres and the central Cu atom are shown. Our optimization with the Spartan software package gives similar structural parameters^{13,14}.

A nitrogen p_π orbital ($2a_{1u}$, Fig. 3a) is close to the HOMO. As schematically shown in Fig. 3c, the HOMO of the CuPc will be shifted up by 7.2 eV, due to the Coulomb repulsion from the four negative charges on the peripheral $-\text{SO}_3^-$, giving rise to a -0.9 eV ($6.3 - 7.2$ eV) binding energy. This estimated binding energy is in agreement with the experimental observation, despite the qualitative nature of the procedure. In the trianion, the most likely protonation position is on the $-\text{SO}_3^-$ group at position 1 (Figs 2 and 3b). The Coulomb repulsion at the central Cu due to the three charges (at positions 2, 3, and 4) is now reduced to 5 eV ($\Sigma_{i=2}^4 e^2/R_{i5}$), again in agreement with the experimental observation that the trianion has a positive binding energy of 1.2 eV. Therefore, we are seeing a stepwise change of the HOMO of CuPc due to the charging at its periphery. Semiempirical calculations using the SPARTAN software package¹³ (which uses the PM3 and PM3TM hamiltonians¹⁴) show that indeed there is a stepwise rigid shift of the molecular orbitals of CuPc due to the negative charges and predicts a negative binding energy for the HOMO of the tetra-anion (K. Ferris, X.-B.W. & L.-S.W., manuscript in preparation). The calculations further confirm that there is little mixing between the molecular orbitals of $-\text{SO}_3^-$ and those of CuPc, and that the charges are indeed localized on the $-\text{SO}_3^-$ groups.

Electrons are always bound in a neutral atom or molecule by the Coulomb force due to the positive core. Relatively few neutral molecules can bind more than one extra electron. A negative electron-binding energy has, to our knowledge, not been observed before, because it cannot exist in neutral or singly charged species. It can only happen in multiply charged anions due to the strong Coulomb repulsion among the excess negative charges. Unlike photoionization of a neutral species (or singly charged anion) where the outgoing electron always experiences a long-range attractive force (Fig. 3a), photoemitted electrons from multiply charged anions experience a long-range Coulomb repulsive force. The short-range electron binding and the long-range Coulomb repulsion create a potential barrier against electron loss in multiply charged anions^{1-3,5} (Fig. 3b and c). Therefore, even when a photon energy is higher than the electron binding energies in a multiply charged

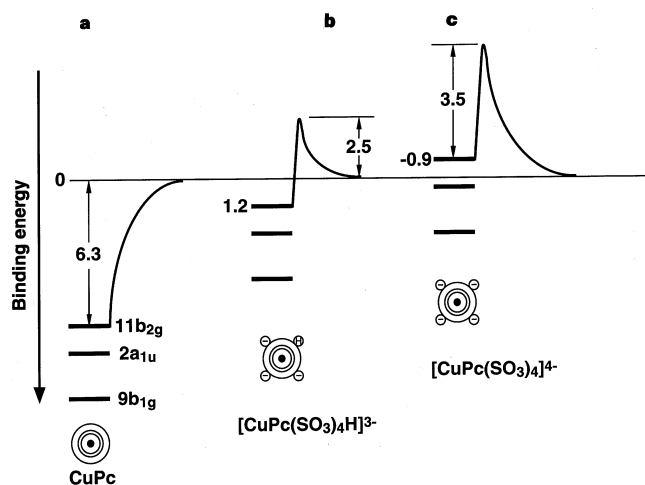


Figure 3 Schematic diagrams showing the top three molecular orbitals of the parent CuPc molecule and the rigid upshift of these orbitals due to the addition of three and four negative charges. **a**, Parent CuPc; **b**, $[\text{CuPc}(\text{SO}_3)_4\text{H}]^{3-}$; **c**, $[\text{CuPc}(\text{SO}_3)_4]^{4-}$. The 'cartoons' show the cyclic neutral CuPc molecule and the sequential charging at its periphery. Schematic potential-energy curves for removing an electron from the highest occupied orbital of the respective species are shown, illustrating the long-range attraction in the CuPc case (**a**), and both the Coulomb barrier and the negative electron-binding energy in the tetra-anion. The experimentally determined electron-binding energies and the Coulomb barrier heights (in eV) are indicated. The 6.3-eV ionization potential of CuPc is from ref. 10.

anion, photoelectrons may not be observed due to the RCB^{6,7}, as demonstrated in the 266-nm spectra of the tri- and tetra-anion (Fig. 1). We estimate that the tri- and tetra-anion possess a barrier height of 2.5 and 3.5 eV, respectively, as shown in Fig. 3. The tetra-anion with a negative electron-binding energy is metastable, and in fact stores 0.9 eV excess electrostatic energy. We can estimate the lifetime of this metastable anion with our ion-trap⁹. We observe no measurable ion loss within the 400 s that we can store the ions. The long lifetime of this metastable tetra-anion in the gas phase is attributed to the large barrier height and the large size of the molecule; the latter means that the electron has to tunnel a long distance to escape. The repulsive Coulomb barrier can be viewed as an electrostatic 'corral' in this semiplanar molecule, trapping the negatively bound electrons inside. These anions can thus be viewed as an electrostatic energy storage medium in the gas phase or a 'molecular capacitor'. They can also be considered as a molecular analogue of a bulk charged metal surface, where the workfunction combined with the electric field around the metal surface creates a potential barrier (the Schottky effect)¹⁵, similar to that shown in Fig. 3c. The RCB in multiply charged anions is analogous to the Coulomb barrier experienced in the α -decay of radioactive nuclei (see, for example, ref. 16). Similar Coulomb barriers against molecular fragmentation exist in multiply charged molecular ions (both anions¹⁷⁻¹⁹ and cations²⁰⁻²²), and provide dynamic stability to such species. □

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Dual modes of the carbon cycle since the Last Glacial Maximum

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The most conspicuous feature of the record of past climate contained in polar ice is the rapid warming which occurs after long intervals of gradual cooling. During the last four transitions from glacial to interglacial conditions, over which such abrupt warmings occur, ice records indicate that the CO_2 concentration of the atmosphere increased by roughly 80 to 100 parts per million by volume (refs 1–4). But the causes of the atmospheric CO_2 concentration increases are unclear. Here we present the stable-carbon-isotope composition ($\delta^{13}\text{CO}_2$) of CO_2 extracted from air trapped in ice at Taylor Dome, Antarctica, from the Last Glacial Maximum to the onset of Holocene times. The global carbon cycle is shown to have operated in two distinct primary modes on the timescale of thousands of years, one when climate was changing relatively slowly and another when warming was rapid, each with a characteristic average stable-carbon-isotope composition of the net CO_2 exchanged by the atmosphere with the land and oceans. $\delta^{13}\text{CO}_2$ increased between 16.5 and 9 thousand years ago by slightly more than would be estimated to be caused by the physical effects of a 5°C rise in global average sea surface temperature driving a CO_2 efflux from the ocean, but our data do not allow specific causes to be constrained.

Carbon dioxide in the atmosphere causes a radiative forcing second only to water vapour, and so may be a central agent in climate change. Polar ice cores contain a detailed record of climate-

related variables which extends more than 400,000 years into the past⁴, and are especially valuable for studying variations of atmospheric CO_2 as they contain an effectively direct record, unlike atmospheric proxies such as marine carbonates or preserved organic material⁵ which may also reflect biological effects. Ice cores from Antarctica, such as those of Taylor Dome and Vostok, are the best sources for palaeoatmospheric CO_2 measurements because they contain only small amounts of the kinds of chemical impurities, such as carbonate dust or organic acids, that may lead to contamination by *in situ* CO_2 production^{2,6–8}. Taylor Dome ice has the additional advantage that the entire 554-m-long core is from above the depth at which air trapped in bubbles in the ice forms gas-ice clathrates. This feature is important because, as we have found, the accuracy of $\delta^{13}\text{CO}_2$ measurements performed on bubble-free ice may be affected by the presence of clathrates. ($\delta^{13}\text{C}$ is defined in Methods.) We have measured the CO_2 concentration and $\delta^{13}\text{CO}_2$ of air trapped in ice from Taylor Dome, Antarctica, across the last glacial termination in order to develop a time series for the carbon-isotope composition of atmospheric CO_2 and to constrain the mechanisms of carbon cycling between the main sources and sinks of atmospheric CO_2 .

The atmospheric CO_2 concentration data^{3,9} (Fig. 1) form a record comparable to that from Byrd and Vostok^{1,2}. The salient features include a low but variable concentration of atmospheric CO_2 between 27.1 and 18.0 kyr BP (thousand years before present, where “present” is defined as AD 1950), when it varied between 186 and 201 parts per million by volume (p.p.m.v.); a rise, at a fairly constant rate, from 190 p.p.m.v. at 17.0 kyr BP to an early Holocene local maximum of 268 p.p.m.v. at 10.6 kyr BP, punctuated by a shallow minimum at 16.1 kyr BP and a drop of 8 p.p.m.v. between 14 and 13 kyr BP which may be related to the Antarctic Cold Reversal³; an 8 p.p.m.v. decrease, from 268 p.p.m.v. at 10.6 kyr BP to 260 p.p.m.v. at 9.1 kyr BP, followed by a rise to the pre-industrial level of about 280 p.p.m.v. (as discussed in detail by Indermühle *et al.*⁹). These data can be combined with the carbon-isotope composition of the CO_2 to give a better picture of how the carbon cycle operated during this time.

The main features of the carbon-isotope record shown in Fig. 1 include a 0.5‰ drop in $\delta^{13}\text{CO}_2$ between 18.0 and 16.5 kyr BP, and the subsequent increase of 0.7‰, from -7.0 ‰ to -6.3 ‰, between 16.5 and 9.1 kyr BP, which is interrupted by two local $\delta^{13}\text{CO}_2$ minima at approximately 13 and 10 kyr BP. The minimum-to-maximum $\delta^{13}\text{CO}_2$ increase began 1,000 to 2,000 years after CO_2 began to rise, and continued to rise for $\sim 3,000$ years after the CO_2 concentration maximum at 10.6 kyr BP, which we interpret as a consequence of the bimodality of carbon cycling (discussed below) rather than a decoupling of CO_2 concentrations from carbon-isotope compositions. Furthermore, there appears to have been an extended local minimum in $\delta^{13}\text{CO}_2$ between 13.4 and 12.3 kyr BP that accompanied the Younger Dryas¹⁰ but was not concurrent with the CO_2 concentration drop at 14 kyr BP. The 0.16‰ difference between the average $\delta^{13}\text{CO}_2$ values of the Last Glacial Maximum (LGM) and Holocene (see Fig. 2 legend for an explanation of these groupings) is similar to the increase of 0.19 ± 0.18 ‰ estimated by Leuenberger *et al.*¹¹ to have occurred between the period 20–40 kyr BP and the early Holocene. These data show, however, that there was a significant drop in $\delta^{13}\text{CO}_2$ between the end of the LGM and the beginning of the transition, and that the subsequent rise into the early Holocene was much larger than the difference between average values for the LGM and Holocene. Moreover, inferences about the carbon cycle drawn from the comparison of the LGM and Holocene averages may be misleading because there was significant variability within each of those periods, and the averages depend strongly on the interval chosen to calculate them. Finally, the variability of $\delta^{13}\text{CO}_2$ before 17.5 kyr BP and after 10.3 kyr BP, 0.33‰ and 0.37‰, respectively, is about half of the magnitude of the

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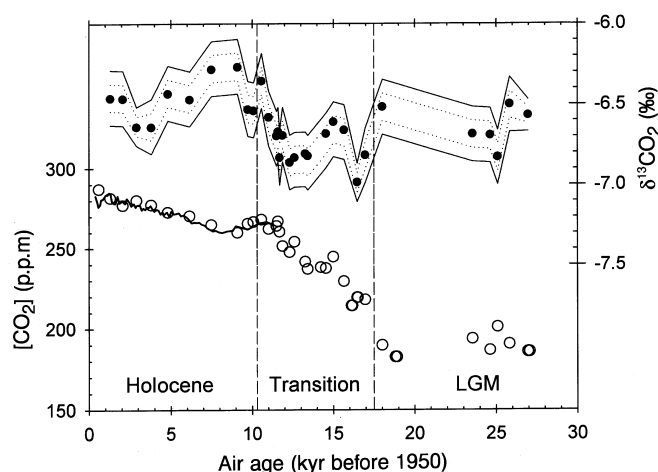


Figure 1 $\delta^{13}\text{CO}_2$ and CO_2 -concentration trends. The data are based on measurements of air trapped in ice from Taylor Dome, Antarctica, and are plotted against air age. Upper curve, $\delta^{13}\text{CO}_2$ values (filled circles) within envelopes of 1σ and 2σ uncertainty (indicated as dotted and solid lines, respectively; see the Methods section for details). Lower curve, CO_2 concentration data shown as open circles were measured in our laboratory at SIO³, while the solid line shown in the Holocene (for comparison) is the higher-resolution record of Indermühle *et al.*⁹. The age axis is divided into three intervals, called "LGM", "transition", and "Holocene" on the basis of CO_2 concentrations and generally accepted ages for these periods. The LGM group includes all of the points with air ages between 27.1 and 18.0 kyr BP, the youngest being the last with a CO_2 concentration below the highest value of the older samples. The transition group includes the points with ages between 17.0 and 10.6 kyr BP, where the local CO_2 maximum which we use to define the start of the Holocene occurs. The Holocene group includes all the points with ages between 10.1 and 1.3 kyr BP (the youngest sample measured for $\delta^{13}\text{CO}_2$). The boundaries between the LGM and the transition, and between the transition and the Holocene, chosen here to fall at 17.5 and 10.3 kyr BP, respectively, are shown as vertical dashed lines.

increase that occurs during the transition, indicating that carbon cycling also varied considerably over shorter timescales.

The atmospheric portion of the carbon cycle is extremely dynamic; ~20% of the CO_2 in the atmosphere is exchanged with the ocean and terrestrial biosphere every year (ref. 12), so the atmosphere responds very quickly to changes in CO_2 cycling between these three reservoirs. The exchange of carbon can affect the isotopic composition of atmospheric CO_2 for two reasons. First, the reservoirs have different carbon-isotope compositions (the $\delta^{13}\text{CO}_2$ of the ocean is close to 0‰, the terrestrial biosphere has a $\delta^{13}\text{CO}_2$ of approximately -25‰ and that of pre-industrial atmosphere was approximately -6.5‰; ref. 13). Second, each flux of CO_2 between the atmosphere and either the ocean or the terrestrial biosphere is accompanied by a different carbon-isotope fractionation, resulting in isotopically unique values of $\delta^{13}\text{CO}_2$ for each transfer. Consequently, variations in the carbon-isotope composition of atmospheric CO_2 can be caused by a net flux of carbon between reservoirs, or by a change in the isotopic fractionation associated with a particular atmospheric flux. It also follows from this that the carbon-isotope composition of the net atmospheric CO_2 exchanged, $\delta^{13}\text{CO}_2(\text{ex})$, is a function of the relative strengths of processes which transfer CO_2 into and out of the atmosphere, so different $\delta^{13}\text{CO}_2(\text{ex})$ values indicate different balances of processes. When a unique balance of processes persists over thousands of years, as indicated by a unique $\delta^{13}\text{CO}_2(\text{ex})$, we refer to it as a 'mode' of the carbon cycle.

In order to examine more closely the implications of the time series shown in Fig. 1, it is advantageous to consider the three data groups (LGM, transition and Holocene) individually. Doing so in

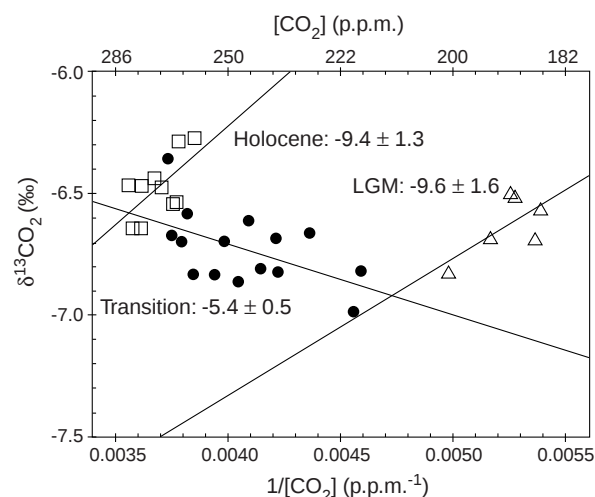


Figure 2 Mixing diagram for Taylor Dome samples: $1/[\text{CO}_2]$ is plotted against $\delta^{13}\text{CO}_2$. The data are divided into three groups, as described in the figure: the LGM group (open triangles, 6 $\delta^{13}\text{CO}_2$ points), the transition group (filled circles, 15 $\delta^{13}\text{CO}_2$ points), and the Holocene group (open squares, 10 $\delta^{13}\text{CO}_2$ points). The y -intercept of the regression line through each of the three groups of data, along with the corresponding 1σ uncertainty for each value, indicates the $\delta^{13}\text{C}$ of the CO_2 exchanged by the atmosphere, $\delta^{13}\text{CO}_2(\text{ex})$, and is shown on the figure.

Fig. 2 reveals a relationship between the concentration of atmospheric CO_2 and its carbon-isotope composition during each of these intervals. (Figure 2 is a mixing diagram, where the inverse CO_2 concentration is plotted against $\delta^{13}\text{CO}_2$, so that addition or removal of CO_2 with a given $\delta^{13}\text{C}$ would appear as a nearly straight line with the y -intercept approximately equal to the isotopic composition of the net CO_2 added to, or subtracted from, the atmosphere.) The coherent trends displayed by these groups show that the carbon cycle has operated in two distinct modes over the past 27 kyr, one during the Holocene, and possibly the LGM as well, when the average $\delta^{13}\text{C}$ of net CO_2 exchanged was approximately -10‰, and another during the transition, when the average $\delta^{13}\text{C}$ of net CO_2 exchanged was approximately -5‰. In other words, during the period of relatively stable, slowly changing climate of the Holocene, the carbon of the net exchanged atmospheric CO_2 was isotopically lighter than the average atmospheric value (that is, $d\delta^{13}\text{CO}_2/d\text{CO}_2$ was negative). In contrast, during the period of rapidly changing climate of the transition the net exchanged CO_2 was isotopically heavier ($d\delta^{13}\text{CO}_2/d\text{CO}_2$ was positive). Therefore, these data suggest that the carbon cycle has operated in two primary modes over the past 27 kyr, one when climate is changing only slowly and another when rapid warming occurs, and that although there are clearly other processes which have affected atmospheric CO_2 over the past 27 kyr, as is evident from the scatter in the data shown in Fig. 2, those transient variations did not overwhelm the longer-term trends.

Many schemes have been advanced to account for the magnitude of the rise in the concentration of atmospheric CO_2 during deglaciations (see, for example, Broecker¹⁴ for a critical review), although none seem to be uniquely sufficient. Two factors which must be included in any analysis of the glacial-interglacial increase in atmospheric CO_2 content, however, are a rise in sea surface temperature (SST) and a decrease in ocean salinity (S). In the range of SST, atmospheric CO_2 concentration and salinity appropriate for this study, changes in SST affect both atmospheric p_{CO_2} and $\delta^{13}\text{CO}_2$, by approximately 4.2% per °C (ref. 15) and 0.11–0.13‰ per °C (ref. 16), respectively, while changes in salinity alter p_{CO_2} by 10 p.p.m.v. per ‰ without affecting its $\delta^{13}\text{C}$ (ref. 17). Measurements performed on tropical corals show that SST increased by 5–6 °C from

the last peak-glacial period to the early Holocene¹⁸, significantly more than the earlier estimate of 1–2 °C by CLIMAP¹⁹, while the change in ocean salinity, estimated from sea level change²⁰, was approximately –1.4‰. Assuming a global average Δ SST (sea surface temperature change) of 5 °C, the combined effects of temperature and salinity changes should have resulted in increases in the concentration and $\delta^{13}\text{CO}_2$ of roughly 30 p.p.m.v. and 0.6‰, respectively. If this were true, then all other processes which caused variations in atmospheric CO_2 must together have increased its concentration by ~50 p.p.m.v. and its $\delta^{13}\text{CO}_2$ by only 0.1‰. A 2 °C global average Δ SST, on the other hand, would have caused no significant change in the concentration of atmospheric CO_2 and increased $\delta^{13}\text{CO}_2$ by roughly 0.2‰, leaving unexplained an approximately 80 p.p.m. concentration increase and a 0.5‰ $\delta^{13}\text{CO}_2$ increase. Adding these changes to the 0.3‰ whole-ocean $\delta^{13}\text{CO}_2$ increase which is thought to have accompanied deglaciation²¹, the expected increase in atmospheric $\delta^{13}\text{CO}_2$ would be between 0.9‰ and 0.6‰, depending on whether a Δ SST of 5 or 2 °C is assumed. The observed increase of 0.7‰ would seem to imply, then, that it is not necessary to invoke a large decrease in surface ocean productivity (which would lower atmospheric $\delta^{13}\text{CO}_2$ to values more negative than are observed) to explain the $\delta^{13}\text{CO}_2$ increase during the transition. What does become necessary, then, is to explain the 0.5‰ drop seen between 18 and 16.5 kyr BP.

In the broadest sense, the atmospheric $\delta^{13}\text{CO}_2$ record presented here points to the ocean as the predominant source of atmospheric CO_2 during the transition. This interpretation follows from two observations. First, even if a change in average global SST alone had caused the increase in $\delta^{13}\text{CO}_2$, a realistic Δ SST would have resulted in an increase of atmospheric CO_2 concentration of less than half of what is observed, so there must have been other sources of CO_2 during that time. Second, because $\delta^{13}\text{CO}_2$ was increasing at a rate equal to or greater than that which rising SST alone would have caused, the additional net CO_2 flux must have had a $\delta^{13}\text{CO}_2$ equal to, or greater than, that of the coexisting atmosphere, eliminating the terrestrial biosphere as a possible source. The most likely explanation for this is an enhanced flux from the ocean (with a $\delta^{13}\text{C}$ close to that of the atmosphere) which transferred CO_2 into the atmosphere at a rate greater than the concurrent uptake of isotopically light CO_2 by an expanding terrestrial biosphere. Unfortunately, the constraints imposed by these data are insufficient to allow the identification of a specific cause for the increased flux of CO_2 from the ocean to the atmosphere. A better understanding of the carbon cycle depends not only on better models, but on additional experimental constraints on the carbon system, particularly more precise data about changes in SST, the size and composition of the terrestrial biosphere, the growth of coral reefs during periods of sea level rise, marine productivity and the chemical composition of the oceans. □

Methods

All samples were taken from Taylor Dome, Antarctica (77°04' S, 158°43' E, elevation 2,374 m), drilled in 1993/94. The methods used for determining the CO_2 concentration of air trapped in ice and the measurement of its carbon-isotope composition are described in detail elsewhere^{22,23}. CO_2 concentration measurements have an internal precision of ± 3 p.p.m.v. (2σ) and are calculated by comparison to three standards of precisely known compositions which are run with every sample. The Craig-corrected²⁴ carbon-isotope measurements, performed on our VG Prism II isotope ratio mass spectrometer, have a 1σ precision of $\pm 0.075\text{‰}$, based on numerous analyses of the CO_2 separated from an atmospheric air standard exposed to uncrushed ice, in order to simulate the conditions of a sample run and to check for fractionation during extraction. $\delta^{13}\text{CO}_2$ is reported in normal δ notation as the per mil difference between the isotopic composition of the sample and standard VPDB carbon, $[(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{VPDB}} - 1] \times 1,000$.

The $\delta^{13}\text{CO}_2$ values reported here have been corrected for the gravitational separation of gases of different masses in the firm, and for the presence of N_2O (which results in isobaric interferences with CO_2 during mass spectrometry).

Gravitational separation in the firm^{25,26} was determined by using the values of $\delta^{15}\text{N}_2$ of air trapped in Taylor Dome ice from Sucher²⁷, following the approach of Sowers and Bender²⁸. The gravitational correction for $\delta^{13}\text{CO}_2$ is ~0.005‰ per m. C-isotope data are corrected for the presence of N_2O on the basis of calibrations performed in our laboratory on CO_2 – N_2O mixtures. The N_2O concentrations of atmospheric air used for this correction, adapted from Leuenberger and Siegenthaler²⁹, are linear interpolations of the following concentrations and dates: 275 p.p.b. (0–9.25 yr BP), 200 p.p.b. (16.1–27.2 kyr BP). The N_2O correction is ~0.001‰ per p.p.b. of N_2O . The total 1σ uncertainty in the reported $\delta^{13}\text{CO}_2$ of a single sample, including uncertainties in the gravitational and N_2O corrections, is 0.085‰. Duplicate analyses of samples with air ages of 2.19 and 17.2 kyr BP have a 1σ uncertainty of 0.060‰ and a triplicate analysis of the sample with an air age of 27.4 kyr BP has a 1σ uncertainty of 0.049‰. The depth–age scale and air age–ice age differences were calculated using a combination of flow modelling, correlating variations in the $\delta^{18}\text{O}$ of the ice with the well-dated GISP2, and matching atmospheric CH_4 concentrations and $\delta^{18}\text{O}$ of O_2 with variations seen in GISP2³⁰.

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Surface effects of bottom-generated turbulence in a shallow tidal sea

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Turbulence in shelf seas strongly affects the spread of pollution (such as oil spills¹) as well as the distribution of sediment² and phytoplankton blooms³. Turbulence is known to be generated intermittently close to the sea bed⁴, but little is known of its evolution through the water column, or to what extent it affects the surface. Here we present observations of the surface effects of bottom-generated turbulence in a tidally influenced and well mixed region of the North Sea, as derived from acoustic and visual images. Although the sea bed in the area is flat, we find that at any one time, 20–30% of the water surface is affected by boils—circular regions of local upwelling—of diameter 0.9 ± 0.2 times the water depth. The signature of individual boils persists for at least 7 minutes and, in accordance with laboratory^{5,6} and numerical⁷ studies, shows the appearance of eddies. The boils contribute to the replacement of surface waters from depth in unstratified waters, and may therefore enhance the fluxes of gases

between atmosphere and ocean.

There are no reported observations of the surface signature of boils in well mixed, open sea. It is, however, clear from dynamical measurements made using current meters that tidal flows generate turbulence through the action of shear stress at the sea bed in much the same manner as in channels in laboratory experiments^{4,8}. Fluid is intermittently ejected away from the bottom in turbulent bursts that may reach vertical speeds of 25% of the forcing current⁴. Numerical⁷ and laboratory^{5,6} studies show that the upward-moving water produces a boil as it impinges on the water surface.

Upward-pointing side-scan sonar has been used to observe a wide variety of processes in the upper ocean^{9–13}. In experiments designed to study the processes leading to dispersion of an oil plume in the tidally well mixed—and consequently unstratified¹⁴—southern North Sea, a two-beam side-scan sonar system was mounted on a frame set on the sea bed at a depth of 45 m. The sonars operate at 80 kHz and 90 kHz, are set at 90° apart in the horizontal, and produce vertical fan-like beams with axes aligned upwards, 20° from the horizontal¹⁵. The site is 56 km from the shore and the local sea bed is flat, with no sand banks or other notable bed forms. The principal acoustic scatterers are bubbles, of typical diameter 20–200 μm , produced in clouds by wind waves as they break¹⁶. The clouds are detectable to ranges of ~ 150 m along the sea surface from the sonar, bubbles accumulating in regions of surface convergence and downwelling^{17,18}. Oil reduces wave breaking and acoustic scatter from the sea surface¹⁰. Acoustic observations were made, however, at least 0.5 km from the oil plume, and in a variety of wind speeds from near calm to 14 m s^{-1} and in tidal currents reaching 1 m s^{-1} .

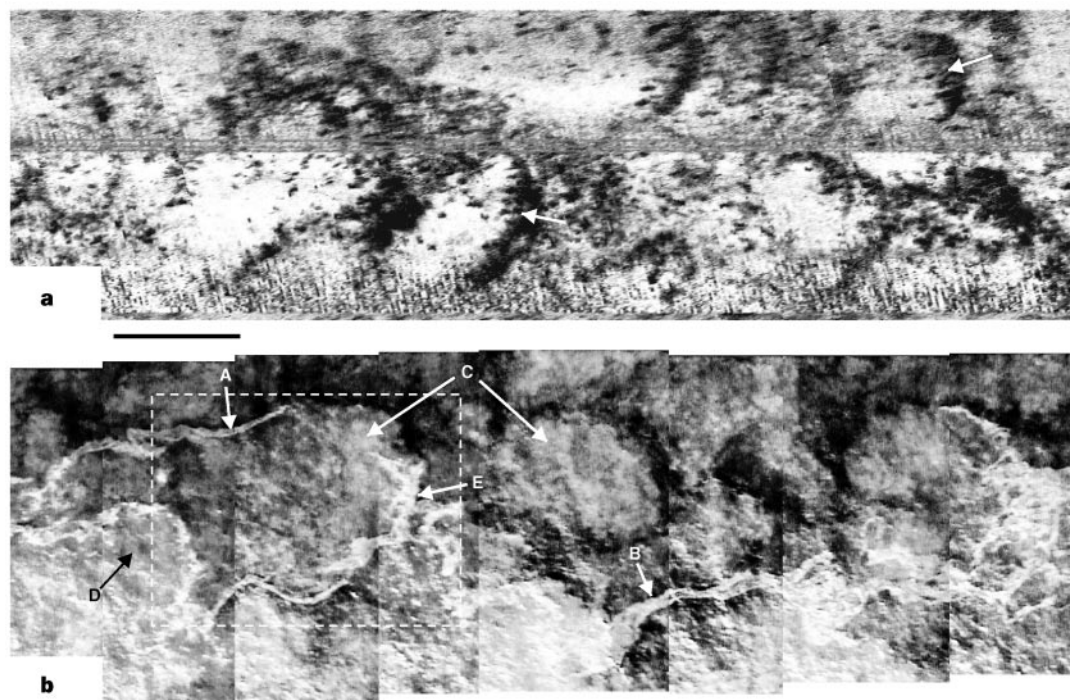


Figure 1 Sonar and video images of boils at the sea surface. The scale is the same in both images (and along both axes), and the tidal current is from right to left; scale bar, 50 m. **a**, Sonograph from a side-scan sonar beam directed across the tidal current showing crescent-shaped dark patterns (as, for example, shown by arrows) from which sound scattering is intense. The mean flow is $0.98 \pm 0.03 \text{ m s}^{-1}$ at 17 m depth and $0.75 \pm 0.02 \text{ m s}^{-1}$ at 33 m depth measured by moored vector-averaging current meters. The tidal current changed by less than $0.1 \text{ m s}^{-1} \text{ h}^{-1}$. The wind 10 m above the sea surface is $6.4 \pm 0.4 \text{ m s}^{-1}$, headed $43 \pm 10^\circ$ left of the current. The image is derived over a 10-min period, 3.5 h after slack water, and as the features shown are being advected through the sonar beam, the timescale has been accordingly converted into distance using their advective speed, 0.89 m s^{-1} , as measured by the sonar directed into the current. The band $\sim 65 \text{ m}$

from the lower edge results from electrical interference. The faint pattern of near-vertical bands at the lower edge results from reflections from surface waves, of period 3.4 s. **b**, Composite image from video of the oil plume being advected from right to left. Oil appears white or nearly white; in this location, $\sim 1.6 \text{ km}$ from the source of the plume, the oil has a filamentary structure (for example, as shown at 'A' and 'B'). Sun-glint is affecting the image along the lower edge, and bright speckles in the centre are caused by sunlight reflecting from surface waves. Lighter blobs show patches of particulate material surrounded by darker regions of clearer water (for example, at positions 'C'). Feature 'D' and the development of the plume within the dashed box are discussed in Fig. 2 legend; oil at 'E' has dispersed laterally around a boil (that is, normal to the mean direction of plume advection).

In low tidal flows and winds exceeding $\sim 4 \text{ m s}^{-1}$, the dominant features observed in the sonograph images of the sea surface are the well-known^{9,11,15,18} linear bands of bubble clouds aligned close to the wind direction in the convergence zones created between neighbouring Langmuir cells. In stronger tidal currents, however, the linear bands were found to be replaced by crescent-shaped features (Fig. 1a). They have a mean diameter of $42 \pm 10 \text{ m}$ ($\pm$ implies one standard deviation) or $(0.93 \pm 0.22)H$, where H is the water depth. The crescents are almost semicircular and are advected with the current. The mean number of features with centres passing through unit length of the sonar beam in unit time is $N = (9.4 \pm 3.3) \times 10^{-5} \text{ m}^{-1} \text{ s}^{-1}$. The sonar beam pointing into the current shows the features to be drifting down-current through the beam at speeds of $0.89 \pm 0.09 \text{ m s}^{-1}$. Such speeds are greater than that of the tidal current at 33 m depth ($0.75 \pm 0.02 \text{ m s}^{-1}$) but less than that at 17 m depth ($0.98 \pm 0.03 \text{ m s}^{-1}$); they are also less than the speeds of isolated bubble clouds ($1.29 \pm 0.08 \text{ m s}^{-1}$) seen in the sonar image, which are characteristic of the wind drift and tidal current in the upper 1 m of the water column^{9,13,16}.

Video images of the oil plume and of the surrounding water surface up to 2 km downstream of a fixed, steady source were obtained at 5-min intervals by an overflying aircraft, 1 h before the sonograph shown in Fig. 1a was obtained. Figure 1b is a composite image of frames 'grabbed' from the video. It shows the plume, which has become filamentary, and, in particular, the appearance of local regions where the oil is abruptly dispersed normal to the direction in which it is being advected (for example, within the dashed box). Oil accumulates in a convergence zone at the upstream edge of these roughly circular regions, and spreads to either side (see also Fig. 2a). The mean diameter of these regions is $47 \pm 14 \text{ m}$ or $(1.04 \pm 0.31)H$, and they cover a proportion, $p \approx 0.2$, of the water surface. Features tracked in the oil plume through a sequence of video images decay or are engulfed in new, but similar, features in a time, $\tau = 410 \pm 140 \text{ s}$, given as a lower bound because of the limited time-span of the images. Figure 1b also shows patches of discoloured water of lighter shading than their surroundings (for example, arrowed 'C'). Their mean diameter is $65 \pm 15 \text{ m}$ or $(1.44 \pm 0.33)H$, and they cover $p \approx 0.3$ of the water surface. The number of such features per unit area is therefore $0.3/[(\pi/4)65^2] = 9.0 \times 10^{-5} \text{ m}^{-2}$. The discoloration seems to result from the presence of particulate matter, probably sediment, originating from—and pointing to—a local source at the sea bed. The evolution of these features is also seen in the Compact Airborne Spectrographic Imager (CASI) images of the break-up of an oil patch (Fig. 3).

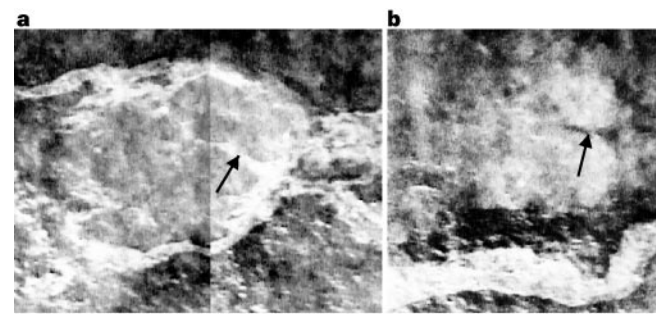
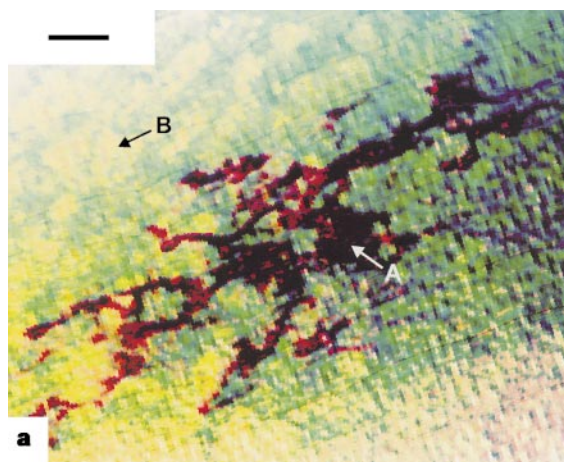


Figure 2 Video images showing details of boils. The tidal current and scales are as for Fig. 1. **a**, The break-up of the oil plume. This is the region within the dashed box at the left of Fig. 1b, but 5 min earlier: abrupt spreading of oil normal to the direction of its advection is evident about a roughly circular sediment patch. By the time of Fig. 1b, the plume at the lower edge of the patch has been distorted by the growth of a new boil, marked therein as 'D'. **b**, A sediment patch lying outside the oil plume (part of which is visible as a white band running across the figure at the bottom). The image shows a characteristic feature, a filament (arrowed), connecting to the upstream side. A similar feature is arrowed in the oil pattern in **a**.

The acoustic and visually observed features have the characteristics of boils often seen in fast-flowing shallow rivers and narrow channels^{19,20}. Boils are produced as upwelling water interacts with, and spreads radially on, the surface^{21,22}. They are often visible in low winds because of their effect in steepening short surface gravity waves²⁰. In laboratory experiments in a channel^{5,6}, dye released near the bottom shows that the boils originate in intermittent ejections from the shear-stress-generated turbulent boundary layer overlying the channel bed. They occur when the upward-moving ejected water impinges on the water surface^{5,6}. Eddies evolve at the edges, and downstream, of the boils⁶. This is consistent with our observations; about 44% of the sediment patches observed in the video images (covering $\sim 0.5 \text{ km}^2$) are divided in two by filaments, $8.6 \pm 2.4 \text{ m}$ in width, roughly aligned with the current (Fig. 2b), suggesting that they consist of pairs of counter-rotating eddies producing an upstream flow along the axis of the features and convergence at their upstream edge. The characteristics of the surface eddies observed in the laboratory, such as separation and size, are found to depend both on outer variables (the free-flow speed, U , and water depth, H) and on inner variables (the friction

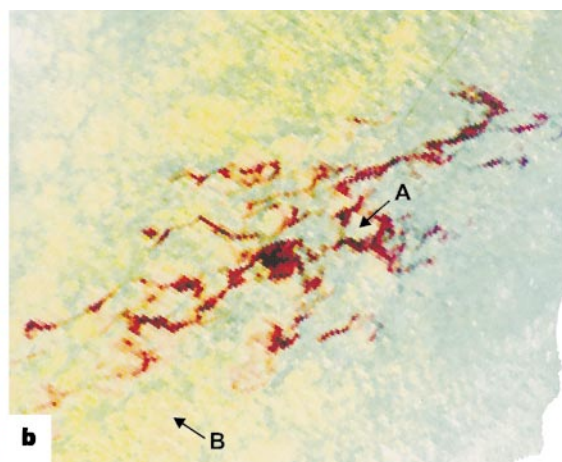


Figure 3 CASI false-colour images of an oil patch (in red). The two images are of the same area of sea surface; **a** was taken 5 min before **b**. Scale bar, 100 m in both images. The oil has dispersed from a patch in the southern North Sea over a period of 4 tides (2 days) in an area of uniform depth, 45 m. The colour scale is different between the images, and **a** has been affected by cloud shadow. Oil thicker than $250 \mu\text{m}$ is imaged as red to black, while yellow and green result from

ambient sea water and oil thinner than $5 \mu\text{m}$. A roughly circular pale blob, of diameter 50 m, is formed within feature 'A' in the oil in the time between the two images. The yellow blobs (for example, at 'B') are clouds of particulates (see also Figs 1b ('C') and 2b). North is to the top. The wind speed 10 m above the sea is 6.3 m s^{-1} from 340° , and the current at 5 m depth is 0.99 m s^{-1} heading to 189° .

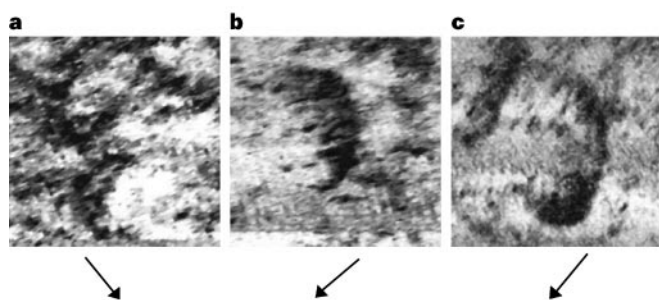


Figure 4 Sonograph images of strongly scattering features (shown as dark) in winds of different directions. The features are represented using the same frozen-field technique described in Fig. 1 legend. In each image, the tidal current is from right to left and the wind direction is shown by an arrow. Each image shows an area 60 m square. The mean current and 10-m wind speed are, respectively, **a**, 0.60 m s⁻¹ (at 19 m depth), 3.0 m s⁻¹; **b**, 0.98 m s⁻¹ (at 17 m depth), 6.3 m s⁻¹; **c**, 0.80 m s⁻¹ (at 15 m depth), 7.1 m s⁻¹.

velocity at the bed, and the kinematic viscosity, ν). The diameter of the laboratory eddies decreases with increasing Reynolds number, $Re = UH/\nu$, being about $3H$ at $Re = 8.8 \times 10^3$, and greater than the size, about H , of eddies marked by sediment in the North Sea. The laboratory Reynolds number is, however, relatively small, ranging from 4.0×10^3 to 8.8×10^3 , whereas Re is about 4.5×10^7 in the North Sea. Whilst the relatively smaller size of the sediment patches may be accounted for by the sinking of sediment (probably faster than 5×10^{-3} m s⁻¹, the settling speed of particulates in the region²), the observation that the laboratory scaling depends on inner variables suggests that the differences in scale may be simply a consequence of the difference in Re .

The boils travel at a velocity different from the combined tidal and wind-drift surface current. As shown by the sonograph images of features found given different relative orientations between wind and tidal current (Fig. 4), in $\sim 90\%$ of cases the acoustic scattering is greatest on the side from which the relative wind-drift comes. The boils are therefore visible in the sonar images because the downwelling, or 'downdrafts'²², at their upwind boundary results in the local accumulation of bubbles^{17,18} and wave steepening²⁰ or even breaking with bubble production, these effects enhancing the scattering of sound there. The surface expression of the boils lags the increasing tidal flow by 100–150 min; this is consistent with other sonar data¹⁵, observations of suspended sediment concentration², and measurements and models of the kinetic energy of developing turbulence^{23,24}. In consequence, boils are only observed at the surface for $\sim 70\%$ of the tidal cycle.

Figures 1b, 2a and 3 demonstrate the dispersive effects of the boils on floating material. Neglecting wind, the horizontal dispersion coefficient normal to a steady tidal current over a smooth bottom is $K = \beta UH$, where β is a constant²⁵. A rough estimate for β can be found from the images. Boils produce a lateral spread of floating particles over scales of order H at times corresponding to those separating the arrival of boils at a given position following the flow. If f is the number of boils produced per unit area per unit time, and τ is their average 'lifetime', then the number of boils reaching the surface per unit area is $f\tau$. If $(1-\epsilon)$ is the fraction of area in which boils overlap and they are advected at speed U , the number of separate boils with centres passing a unit line normal to the flow in unit time is $N = f\tau U$. If A is the area of a single boil then $p = f\tau A\epsilon = AN/U = 0.14 \pm 0.07$ from sonar data (compared to the observed $p \approx 0.2$). The surface area fraction covered per unit time is $fA\epsilon$ and the average time for an area to be covered is $T = (fA\epsilon)^{-1}$, the mean time between boils affecting a given area moving with the mean flow. The dispersion coefficient is $\sim H^2/T$, or $ANH^2/\tau U$, so that $\beta (= ANH/\tau U^2)$ ranges from 6.4×10^{-3} to 3.9×10^{-2} , using the sonar

data and the probable underestimate of τ based on the oil plume. This spans the values, $\beta = (1.0 \pm 0.4) \times 10^{-2}$, found in smooth-bottomed laboratory channels²⁶, though the dependence on Re is uncertain.

The images capture the generation of tidal boils and eddies over a level sea bed, showing that they are likely to be of common occurrence in tidal shelf-seas. They also provide insight into the vertical transport of water, sediment and algae on the shelf, at the larger scales that develop following ejections in turbulent bursts from close to the sea bed. The boils impose a patchy structure in, for example, the colour of the water surface (see Figs 1b, 2 and 3), which may bias the measured average values of sea-surface parameters detected by satellite or other 'remote' sensors. The images illustrate how boils disperse oil patches or plumes. Turbulence generated by the shear stress of tidal flow on the sea bed is evidently an important process of short-term dispersion in tidally mixed shelf seas, at least in wind speeds less than about $15U$ (ref. 27), at scales smaller than the Rossby radius (when rotation becomes significant) and until bathymetry becomes important²⁸; in stronger winds, dispersion resulting from Langmuir circulation¹² may dominate. The appearance of the circles in the oil films (Fig. 3) demonstrates that boils help to replenish surface waters, a process important in gas exchange^{16,29}. Such boils will be absent at the surface of density-stratified seas, where the thermocline separates the surface and the deep water, preventing bursts and ejections generated in the benthic boundary layer from reaching the surface. The intensity of near-surface turbulence and the rate of air-sea gas exchange in deep stratified oceanic waters may in consequence be lower than in well mixed tidal seas. □

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Predation enhances complexity in the evolution of electric fish signals

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Theories of sexual selection assume that predation is a restrictive, simplifying force in the evolution of animal display characters¹ and many empirical studies have shown that predation opposes excessive elaboration of sexually selected traits². In an unexpected turnaround, I show here that predation pressure on neotropical, weakly electric fish (order Gymnotiformes) seems to have selected for greater signal complexity, by favouring characters that have enabled further signal elaboration by sexual selection. Most gymnotiform fish demonstrate adaptations that lower detectability of their electrolocation/communication signals by key predators. A second wave phase added to the ancestral monophasic signal shifts the emitted spectrum above the most sensitive frequencies of electroreceptive predators. By using playback trials with the predatory electric eel (*Electrophorus electricus*), I show that these biphasic signals are less detectable than the primitive monophasic signals. But sexually mature males of many species in the family Hypopomidae extend the duration of the second phase of their electric signal pulses³ and further amplify this sexual dimorphism nightly during the peak hours of reproduction⁴. Thus a signal

element that evolved for crypsis has itself been modified by sexual selection.

Weakly electric fish generate multipurpose electric signals for electrolocation and communication^{5,6}. Anatomical, physiological and developmental evidence together indicate that the ancestral waveform of the electric organ discharge (EOD) was an intermittent monophasic pulse^{5,7–9}. This primitive discharge type is rare in extant gymnotiform fish, having been replaced largely by continuous wave trains (in three families) or multiphasic pulsed waveforms (in three families) (Fig. 1). To address the forces that mould signal complexity, I focus here on the diverse EOD waveforms of pulse-discharging fish. I consider electrolocation, sexual selection and avoidance of predation as possible factors that could favour the switch from a monophasic to a multiphasic EOD.

In fact, electrolocation can favour increased complexity of the EOD, and may well have done so in those species with accessory electric organs and enhanced waveform complexity at the head instead of the tail⁹. But in the biphasic *Brachyhypopomus* species, local biphasy occurs at the tail but not at the head (Fig. 2) where electrosensory exploratory behaviour occurs (P.K.S., unpublished data) and where electroreceptors are most dense¹⁰. Therefore, among the *Brachyhypopomus* species with biphasic waveforms, the simplest example of signal enhancement, electrolocation could not have been involved with the transition from monophasy to biphasy in the main electric organ.

Most *Brachyhypopomus* species display sexual dimorphism in the second phase of their biphasic pulse EODs, particularly at the tail (Fig. 2)^{11–13}. But, before modification for sex recognition and mate attraction^{13,14}, the second phase would have existed probably in some sexually monomorphic form. Furthermore, in other gymnotiform families—such as Gymnotidae, Rhamphichthyidae and Aptereronotidae—this additional phase is present but not sexually dimorphic. Sexual selection thus does support the ancestral conversions from monophasy to biphasy.

Key predators of weakly electric gymnotiforms are pimelodid catfishes and the electric eel^{12,15}. Both the Gymnotiformes and their sister order Siluriformes (catfish) possess ampullary electroreceptors with extreme sensitivity to low frequencies^{16–18}. The ampullary system is specialized for detecting weak electric fields of prey (passive electrolocation) but is tuned below the spectrum of most gymnotiform EODs. The best frequencies for gymnotiform and catfish ampullary receptors are about 30 Hz and 8 Hz (refs 16, 18) respectively, whereas the spectral peaks of gymnotiform EODs are generally much higher, in the range of 50–3,000 Hz (ref. 19). Gymnotiforms, but not catfish, evolved a second parallel electroreceptive pathway, the tuberous electroreceptor system, two

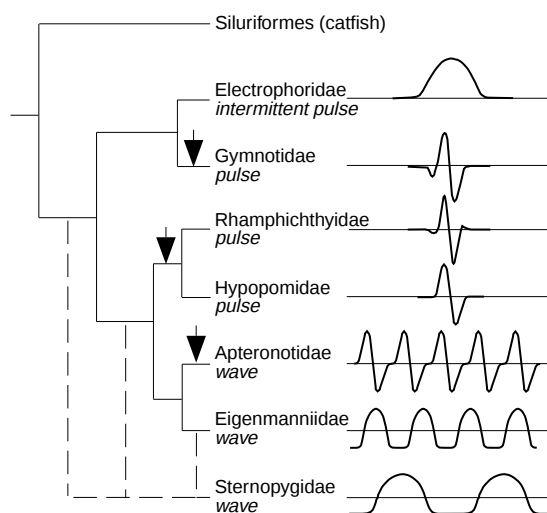


Figure 1 Molecular-morphological consensus phylogeny of the gymnotiform families^{22,30} is plotted along with schematic electric organ discharge (EOD) waveforms of representative species. Arrows depict postulated transitions from simple monophasic to multiphasic EOD waveforms. Dashed lines represent equally supported alternate phylogenies. Aptereronotidae possess independently derived electric organs, thus the biphasic EOD is derived in this family.

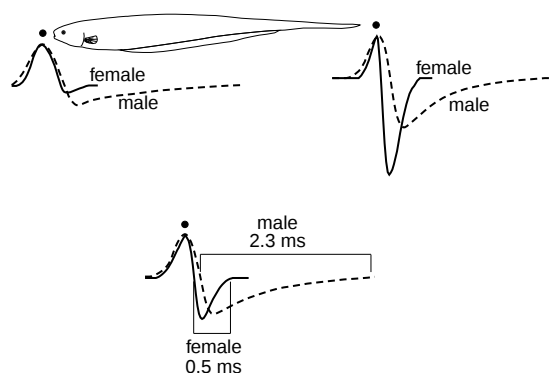


Figure 2 Local and remote EODs of male and female *Brachyhypopomus pinnicaudatus*. EOD waveforms were recorded at locations indicated by the dots. Amplitudes are rescaled here to normalize peaks of the first EOD phase. Note that the EODs are sexually dimorphic, with the male's second phase extended in duration, and that the EOD local to the head is nearly monophasic, particularly in the female.

orders of magnitude less sensitive than the ampullary system, but tuned to the higher frequencies of the EOD^{6,20}. Tuberous electroreceptors sense the autogenous EOD during active electrolocation of nearby objects such as prey, predators, mates and hiding places. EODs and tuberous electroreceptors appear as matched co-adaptations that allow electrolocation above the frequency range of the predator's sensitive ampullary electroreceptors. What adaptations shifted the EOD spectrum upwards?

The ancestral monophasic EOD pulse⁵ resembles a single-period cosine wave which has a low frequency spectral peak close to 0 Hz (Fig. 3b), just where electroreceptive predators are maximally sensitive. Fig. 3 shows that the addition of a negative-going second phase shifts spectral energy upwards, above the best frequency of the ampullary electroreceptors. The transition from monophasic to multiphasic EODs evolved at least twice among pulse families and a third time in a wave family Apterontidae (Fig. 1). The wave fish Sternopygidae, Eigenmanniidae and Apterontidae generate EOD trains with even duty cycles (Fig. 1), resulting in a further narrowing of the spectrum^{19,20}.

Can the shift from monophasy to multiphasy reduce detection by electroreceptive predators? To address this question I obtained a large electric eel and trained it with food to approach electrodes playing a variety of electric playback signals (see Methods). In a randomized series of trials, the eel was presented, at natural intensities, with either the biphasic EODs of female *Brachyhypopomus pinnicaudatus* or the same waveforms rendered monophasic by digital deletion of the second phase (Fig. 3c, d). The electric eel was half as likely to approach the playback of the biphasic EOD as the monophasic half-EOD even though peak-to-peak amplitudes of the biphasic stimuli were twice as great (approach to biphasic EOD in 6/21 trials compared with approach to monophasic EOD in 12/18 trials; Fisher's exact test, $P = 0.01$).

I recorded EODs of three gymnotiform species that emit pulsed

monophasic EODs (Fig. 4a). Each has a unique circumstance that reduces its vulnerability to electroreceptive predators. The first species, the electric eel, is itself an electroreceptive predator and produces a low-voltage, monophasic EOD for electrolocation but can also produce high-voltage discharges (to 600 V) for defence and prey capture²¹. Current phylogenies place the electric eel at or near the root of the order Gymnotiformes, with monophasy primitive, but well defended in all respects²². The second species with a simple monophasic EOD is *Gymnotus cylindricus*, common to streams of middle America. The *G. cylindricus* species group, the northernmost gymnotiform, inhabits a geographic refuge devoid of the two key electroreceptive predator groups, the electric eel and large pimelodid catfishes²³. The third species with a monophasic EOD is a small, defenceless *Brachyhypopomus* from Amazonia, a region rich in electroreceptive predators. A well-resolved phylogeny shows that this monophasic species is derived from a biphasic ancestor³. This species has the largest electric organ in its genus³. Field workers frequently mistake its EOD for that of the sympatric electric eel (J. Alves-Gomes, personal communication). On the basis of her field studies of fish in northern Peru, M. Hagedorn (personal communication) proposed that the monophasic *Brachyhypopomus* is a batesian mimic of the electric eel.

I took calibrated EOD recordings of three specimens of the monophasic *Brachyhypopomus* and compared them with calibrated

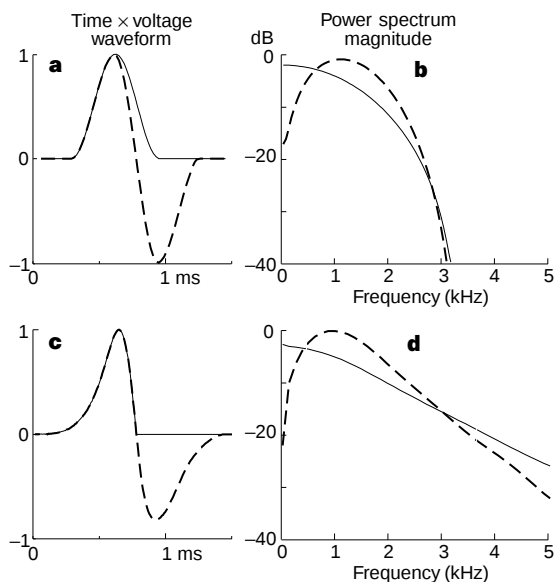


Figure 3 Biphasic EOD pulses have less energy at low frequencies than their monophasic counterparts. **a**, The ancestral monophasic EOD is modelled as a single-period inverted cosine wave (solid line). Addition of a second cosine wave, offset in time, produces a biphasic pulse (dashed line) that **b**, raises the frequency of the spectral peak and depresses spectral amplitudes below 600 Hz. Ampullary electroreceptors of electroreceptive predators are maximally sensitive around 8 Hz (Siluriformes) or 30 Hz (Gymnotiformes), and so the shift to biphasy should reduce detection by predators. **c**, Waveforms and **d**, power spectra of EODs used as playback stimuli with the electric eel: the biphasic pulse from a female *Brachyhypopomus pinnicaudatus* (dashed line) and the first phase only of the same EOD (solid line). In playback trials, the electric eel detected playback of the biphasic EOD less than half as often as when the first phase was delivered alone.

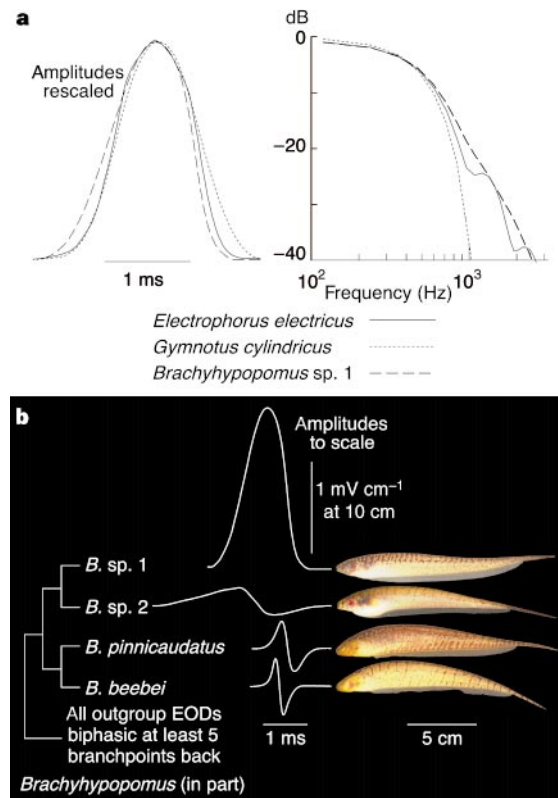


Figure 4 Monophasic EODs of three species compared with each other and with biphasic EODs of congeners. **a**, Power spectra and EOD waveforms of three gymnotiform species with monophasic EOD pulses. The EODs and power spectra of all three monophasic species are extremely similar compared with those of biphasic EODs (Fig. 3). The monophasic *Brachyhypopomus* is proposed to have lost the second phase of its EOD as an adaptation to resemble the dangerous electric eel *Electrophorus*. **b**, Phylogeny³, photographs and EOD waveforms of mature females of four closely related sympatric *Brachyhypopomus* species. All earlier branches in the superfamily show biphasic EODs, thus monophasy in this genus is a derived loss of the second phase. The EOD of monophasic *Brachyhypopomus* sp. 1 is significantly greater in amplitude than similarly sized members of its sister species, another feature that resembles the EOD of the electric eel.

EOD records of its three closest relatives³ (Fig. 4b). The EOD of the monophasic *Brachyhypopomus* species is similar to that of the electric eel in waveform, duration and spectrum²⁴ (Fig. 4a). The resting discharge rate was low (8–13 Hz), resembling an alert electric eel in both rate and variability²⁴. Calibrated daytime EOD amplitudes were 1.5–1.8 mV cm⁻¹ at 10 cm, which is between five and ten times greater than specimens of its sister species³ *Brachyhypopomus* sp. 2 (Fig. 4b). Thus the monophasic *Brachyhypopomus* species appears to have lost the second phase of its EOD and boosted EOD amplitude, consistent with Hagedorn's proposal that this fish is a batesian electric mimic of the sympatric electric eel. Further confirmation of the hypothesis could come only from experiments showing mutual avoidance of electric eels and monophasic *Brachyhypopomus* by an electroreceptive predator.

In summary, predation avoidance is the strongest candidate as the driving force for the initial evolution of EOD complexity, in particular, the transition from primitive monophasy to biphasy. This conclusion is supported by three lines of evidence: (1) spectral comparison of monophasic and biphasic EODs; (2) demonstration that biphasic pulses are less detectable by a known electroreceptive predator; and (3) examples of specific adaptations (high voltage, geographic isolation and mimicry) that protect species with monophasic EODs. Sexual dimorphism in the second EOD phase of *Brachyhypopomus* spp. (Fig. 2) seems to be the secondary modification of an adaptation for signal crypsis. Evolutionary escape from predation has been cited as a key factor promoting adaptive radiation²⁵. Thus spectral shifting may have contributed to the success of this order in tropical South America. A key question is whether signal multiphasy evolved in any gymnotiforms outside the geographic range of their electroreceptive predators. Several extant multiphasic gymnotiform taxa extend beyond the range of large electroreceptive predators²³ (O. Macadar, personal communication), but their centres of distribution lie in the predator-rich continental tropics and none could be argued to represent an independent origin of multiphasy.

A parallel story may emerge from Africa, where mormyrid electric fish have undergone extensive radiation and an electroreceptive predator, the catfish *Clarias*, serves as their major predator^{26,27}. Nor are electric fish entirely unique in having protective signal adaptations exploited by sexual selection. Ctenuchid moths evolved acoustic signals to alert predatory bats of their toxicity, and these signals have likewise been co-opted for mate attraction^{28,29}. □

Methods

An electric eel 1 m long was trained to receive food (goldfish) when it approached any playback of an electric field in its round aquarium (120 cm diameter, 60 cm deep). We played electric stimuli from a DC-coupled 5-cm carbon dipole at calibrated intensities equivalent to natural EODs⁴. DC offset at 10 cm from the dipole centre was less than 0.05 μ V cm⁻¹. Training stimuli included a wide variety of monophasic and biphasic digitized EODs. Experimental stimuli included the biphasic EOD of a female *Brachyhypopomus pinnicaudatus* and the same EOD with the second phase digitally removed (Fig. 3c, d). Trials were sequenced randomly. Playbacks of 1 min duration began while the eel rested on the tank bottom, at a distance of more than 60 cm from the electrode. A 'blind' assistant rewarded all electrotactic approaches with food. In the first set of trials, playback rate simulated a repeated social signal, 0.5 s at 50 Hz alternated with 0.5 s at 200 Hz. In a second set of trials playing the truncated stimulus only, rate modulation had no effect on frequency of approach (19/21 trials with rate modulation compared with 19/20 trials at 50 Hz). I measured EOD amplitudes (Fig. 4b) by methods published previously⁴.

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'Green revolution' genes encode mutant gibberellin response modulators

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World wheat grain yields increased substantially in the 1960s and 1970s because farmers rapidly adopted the new varieties and cultivation methods of the so-called 'green revolution'^{1–4}. The new varieties are shorter, increase grain yield at the expense of straw biomass, and are more resistant to damage by wind and

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rain^{3,4}. These wheats are short because they respond abnormally to the plant growth hormone gibberellin. This reduced response to gibberellin is conferred by mutant dwarfing alleles at one of two *Reduced height-1* (*Rht-B1* and *Rht-D1*) loci^{4,5}. Here we show that *Rht-B1/Rht-D1* and maize *dwarf-8* (*d8*)^{6,7} are orthologues of the *Arabidopsis* *Gibberellin Insensitive* (*GAI*) gene^{8,9}. These genes encode proteins that resemble nuclear transcription factors and contain an SH2-like¹⁰ domain, indicating that phosphotyrosine may participate in gibberellin signalling. Six different orthologous dwarfing mutant alleles encode proteins that are altered in a conserved amino-terminal gibberellin signalling domain. Transgenic rice plants containing a mutant *GAI* allele give reduced responses to gibberellin and are dwarfed, indicating that mutant *GAI* orthologues could be used to increase yield in a wide range of crop species.

Gibberellin is an essential endogenous regulator of plant growth¹¹. *Rht-B1b* and *Rht-D1b* are semidominant, altered function (rather than loss-of-function) mutant alleles of the *Rht-1* height-regulating genes of wheat. These mutant alleles reduce plant height (Fig. 1a), reduce responses to gibberellin and increase in *planta* gibberellin levels^{4,5,12,13}. These properties are also characteristic of

mutant alleles of maize *d8*^{6,7,14} and of the *Arabidopsis* *gai* allele^{8,9,15}, indicating that these mutant alleles might define orthologous genes that are involved in gibberellin signalling. *GAI* (the wild-type allele) encodes a protein (*GAI*) containing features that are characteristic of transcription factors⁹. The *gai* allele encodes a mutant protein (*gai*), lacking 17 amino acids from near the amino terminus, that is thought to confer the altered gibberellin responses characteristic of the *gai* mutant⁹. Database searches revealed a rice expressed-sequence tag (EST; D39460) that encodes a potential polypeptide containing a sequence nearly identical to these 17 amino acids¹⁶. We used this EST to investigate whether the dominant dwarfing mutant alleles of *GAI*, *Rht-1* and *d8* identify orthologous genes in *Arabidopsis*, wheat and maize.

D39460 was used to isolate wheat complementary DNA C15-1. The genome of bread wheat is hexaploid, consisting of three homoeologous chromosome sets (the A, B and D genomes). Analysis of lines lacking particular chromosomes (nullisomic) showed that C15-1 hybridized to genomic DNA fragments derived from wheat chromosomes 4A, 4B and 4D (Fig. 1b), correlating with the location of the *Rht-1* alleles (all known mutant *Rht-1* alleles are on chromosomes 4B or 4D; ref. 5). Furthermore, restriction-

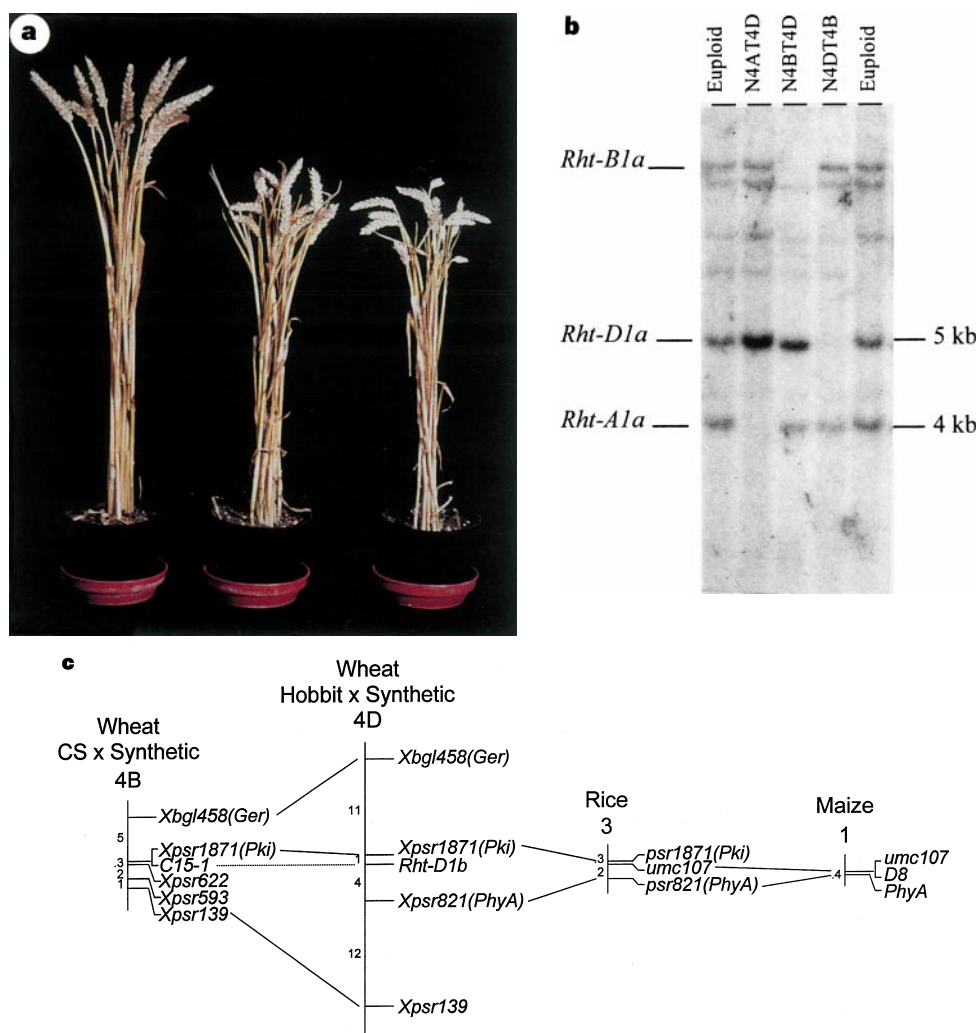


Figure 1 cDNA C15-1 maps to the *Rht-1* locus. **a**, Near-isogenic dwarf wheat lines: left, tall control (var. Mercia); centre, semi-dwarf *Rht-B1b*; right, semi-dwarf *Rht-D1b*. **b**, Gel-blot hybridization of C15-1 with *DraI*-digested DNA from wheat lines lacking individual group 4 chromosomes (nullisomic 4A-tetrasomic 4D, N4AT4D; nullisomic 4B-tetrasomic 4D, N4BT4D; nullisomic 4D-tetrasomic 4B, N4DT4B), and euploid control (all var. Chinese Spring). Hybridizing fragments were assigned to chromosomes (4A, 4B and 4D) as shown. **c**, Partial linkage maps of wheat chromosomes 4B (ref. 26) and 4D, rice chromosome 3 (ref. 28), and maize

chromosome 1 (ref. 29) showing the colinearity between regions containing C15-1, *Rht-D1b* and *D8-1*. A putative maize *d8* genomic fragment (see text) also displayed tight linkage with *umc107* (not shown). Wheat 4B data are from the F₂ of a Chinese Spring (CS) × Synthetic cross. Wheat 4D data are from the F₂ of a Hobbit (contains *Rht-D1b*) × Synthetic cross; segregation for *Rht-D1b* was assayed by seedling responses to gibberellin¹². Map distances are in centi-Morgans (cM).

fragment length polymorphism mapping showed that C15-1 is tightly linked to *Xpsr1871(Pki)*, a marker that is itself tightly linked with *Rht-D1b* (Fig. 1c). Cereal genomes show substantial conservation in gene order (colinearity). The region of wheat chromosomes 4A, 4B and 4D to which C15-1 hybridizes is colinear with a segment of rice chromosome 3, and with a segment of maize chromosome 1 containing *d8* (Fig. 1c)¹⁷. These observations are consistent with the hypothesis that the C15-1 transcript is derived from one of the *Rht-1* homoeoalleles.

We used C15-1 to isolate genomic DNA clones containing the putative *Rht-B1a* and *Rht-D1a* (the *Rht-B1* and *Rht-D1* wild-type alleles⁵) and maize *d8* (the *D8* wild-type allele⁷) genes. The amino-acid sequences of the proteins encoded by wheat *Rht-D1a* (*Rht-D1a*), maize *d8* (*d8*) and *Arabidopsis* *GAI* (*GAI*) and *RGA* (*RGA*) were compared (Fig. 2a; *RGA* is an *Arabidopsis* gibberellin signalling

protein that is closely related to *GAI*; ref. 16). *Rht-D1a* and *d8* appear to be more closely related to *GAI* than they are to *RGA* (per cent amino-acid identity, *GAI* versus *RGA* is, respectively, 62 versus 58% (*Rht-D1a*) and 62 versus 59% (*d8*)). The carboxy-terminal ~2/3 of all four proteins are very similar to each other and to the equivalent region of SCARECROW (*SCR*), a candidate transcription factor from *Arabidopsis*^{9,16,18}, and of LATERAL SUPPRESSOR (*Ls*), a tomato protein required for formation of axillary branches during vegetative growth¹⁹. The N-terminal regions of the *GAI*/*RGA*/*Rht-D1a*/*d8* proteins contain two regions of closely related sequence (regions I and II in Fig. 2a). Regions I and II are found in five gibberellin signalling proteins (*GAI*, *RGA*, *Rht-D1a*, *d8* and also in *Rht-B1a*, the *Rht-B1a* gene product; Fig. 3a), but are not found in *SCR* or *Ls*, indicating that they may be responsible for the gibberellin-specific action of these proteins^{9,16}. Furthermore,

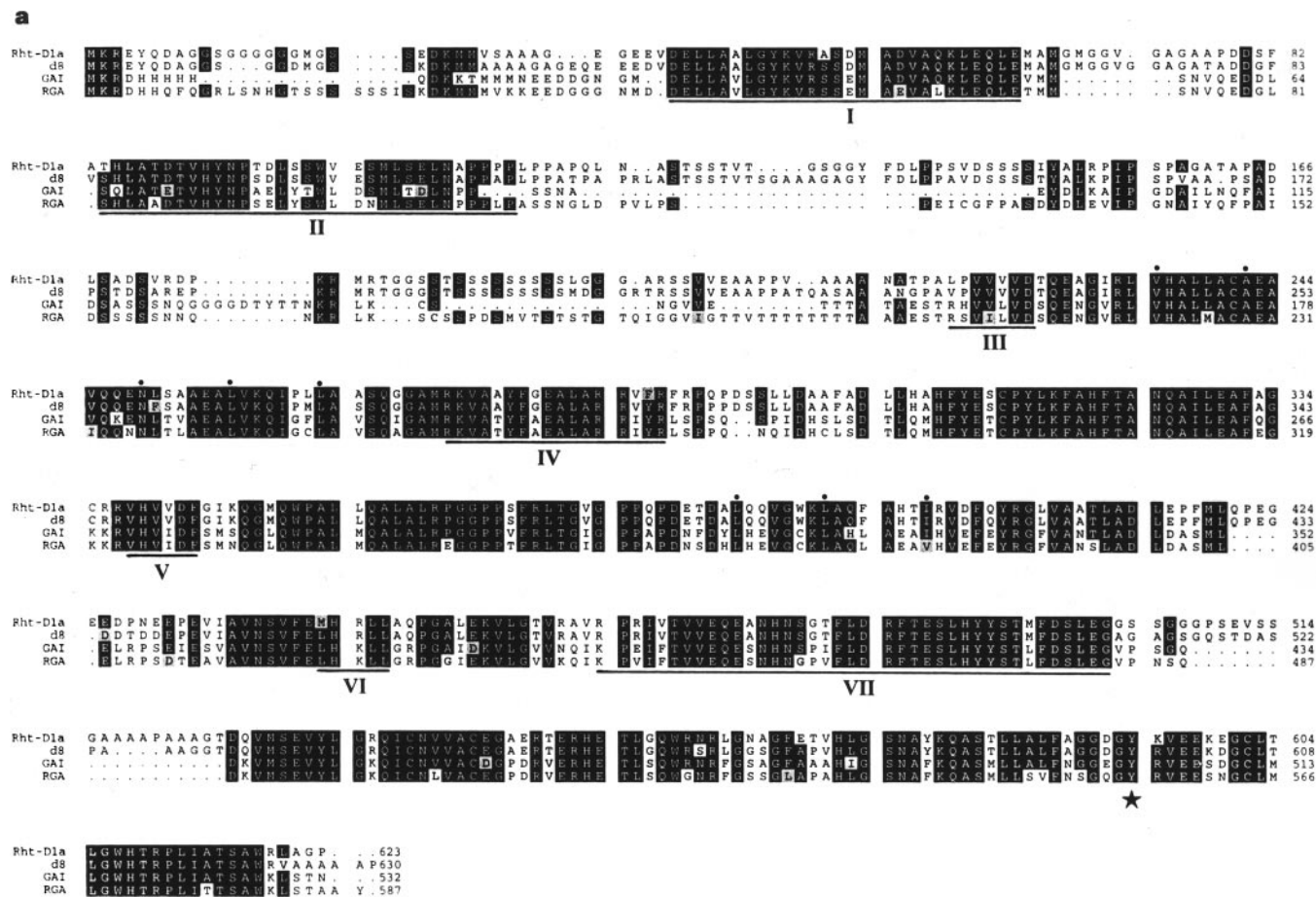


Figure 2 Structural features of *Rht-D1a*, *d8*, *GAI* and *RGA*. **a**, Amino-acid sequence alignment comparing *Rht-D1a*, *d8*, *GAI*⁹ and *RGA*¹⁶. Gaps are introduced to maximize alignment. Exact matches are boxed in black, shaded boxes indicate conservative substitutions. Indicated regions of conserved amino-acid sequence are: I and II, N-terminal regions that are not present in *SCR*¹⁸ or *Ls*¹⁹; III and V, valine-rich regions; IV, nuclear-localization signal^{9,16}; VI, LXXLL motif^{9,16}; VII, SH2-like domain. Residues defining leucine heptad repeats are indicated by closed circles. Star, potential site of tyrosine phosphorylation (this feature, leucine heptad repeats, regions III, V and VII, and the relative positions of all of these features/regions are characteristic of STATs; ref. 22). **b**, Amino-acid sequence alignment comparing sequence from region VII of *Rht-D1a* (residues 464–503)

with those of previously recognized SH2 domains. Functionally significant residues are in bold and italicized. Numbers in parentheses refer to the number of intervening residues that are not shown. A typical SH2 domain is a peptide stretch of 100 amino acids containing an invariant arginine (**R** with asterisk) that recognizes the phosphate group of phosphotyrosine. Following this arginine are other strongly conserved residues (diamonds). These residues, together with another upstream arginine or lysine (**R** or **K** with diamond), interact with the phosphate group, the tyrosine ring, and the polypeptide backbone of the ligand^{20,21}. Other regions of the SH2 domain are less conserved, although their three-dimensional structures are similar. Sequences shown are c-Ab1¹⁰, c-Src¹⁰, PLC-γ1N¹⁰, Dd-Stat³⁰ and Stat5a²¹.

region I is substantially deleted in the *Arabidopsis gai* mutant, confirming that this region is important for gibberellin signalling⁹.

Analysis of the GAI/RGA/Rht-D1a/d8 sequences revealed an SH2-like domain within the C-terminal section of the protein (region VII in Fig. 2; Fig. 2b). SH2 domains are associated with phosphotyrosine signalling in metazoans¹⁰, and bind tyrosine-phosphorylated polypeptides at an essential arginine residue. This residue is invariant in SH2 domains and is found in the GAI/RGA/Rht-D1a/d8 SH2-like domain (Fig. 2a, b). Alignment of the Rht-D1a SH2-like domain with previously identified SH2 domains reveals substantial conservation of the amino-acid sequence, especially of those residues that assist in the binding of the phosphorylated tyrosine to the invariant arginine^{20,21} (Fig. 2b). To our knowledge, this is the first identified putative SH2 domain in plants. STAT (signal transducers and activators of transcription) proteins are transcriptional regulators that contain SH2 domains²². GAI/RGA/Rht-D1a/d8 are candidate transcription factors that contain an SH2-like domain, and display other features characteristic of STATs (for details, see Fig. 2a). Phosphotyrosine signalling may be involved in gibberellin-mediated plant growth regulation, using proteins similar to the STAT factors that mediate cytokine/growth-factor control of growth in animals.

Rht-B1, *Rht-D1* and *d8* are defined by allelic series of semi-dominant mutations that confer differing severities of dwarfism⁴⁻⁷. To identify the molecular basis of these mutations, the DNA sequences of five mutant alleles (*Rht-B1b*, *Rht-D1b* and three *D8* alleles) were determined. Each allele contains a mutation that alters the N-terminal region of the protein that it encodes (Fig. 3a). All three maize mutant proteins (*D8-1*, *D8-2023* and *D8-Mpl*), like *Arabidopsis gai*, lack regions of the peptide sequence. *D8-1* and *D8-2023* are, like *gai*, in-frame deletion mutations. In *D8-1*, D55 is replaced by a glycine, and 56-VAQK-59 are missing. This segment is very close to that deleted in *gai*, and falls within the highly conserved region I (Fig. 3a). *D8-2023* lacks 87-LATDTVHYNPSD-98 from within the highly conserved region II (Fig. 3a). The *D8-Mpl* mutation is a 330-base pair (bp) deletion that extends from the 5' untranslated sequence through the presumed (normal) start ATG codon and ends at V84. Genetic analysis⁶ indicates that *D8-Mpl*, like *D8-1*, makes an active product. Presumably, *D8-Mpl* translation initiates at M106 (or a subsequent methionine), and makes an N-

terminally truncated product that lacks region I and most of region II (Fig. 3a).

The *Rht-B1b* and *Rht-D1b* mutations are both nucleotide substitutions that create stop codons. In *Rht-B1b*, a T-for-C substitution converts the Q64 codon (CGA) to a translational stop codon (TGA; Fig. 3a). In *Rht-D1b*, a T-for-G substitution converts the E61 codon (GGA) to a translational stop codon (TGA; Fig. 3a). The similarity of the *Rht-B1b* and *Rht-D1b* mutations presumably explains why they confer very similar severities of dwarfism⁴. Genetic analysis indicates that both *Rht-B1b* and *Rht-D1b* make active products¹². It is possible that the short N-terminal peptide fragments encoded by *Rht-B1b* and *Rht-D1b* confer the mutant phenotype. However, it is also possible that ribosomal scanning following translational termination at the mutant stop codons in *Rht-B1b* and *Rht-D1b* permits translational reinitiation at one or other of the several methionines that closely follow these stop codons²³, and that the resultant N-terminally truncated product confers the mutant phenotype. This seems more likely, as the *D8-Mpl* allele also encodes an N-terminally truncated product (see above). Thus *Rht-B1b* and *Rht-D1b*, like *D8-Mpl*, apparently encode N-terminally truncated products that lack region I (Fig. 3a).

Mutagenesis of *Arabidopsis gai* can generate apparent loss-of-function derivative alleles which confer a tall, rather than dwarf, phenotype²⁴. These derivative alleles carry mutations that interrupt the *gai* open reading frame (ORF)⁹ and thereby abolish *gai* function. Similarly, following fast-neutron mutagenesis, we obtained an apparent loss-of-function allele derived from wheat *Rht-B1b*. This new allele (*Rht-B1g*) confers a tall, gibberellin-responsive phenotype, rather than the dwarf, gibberellin-resistant phenotype characteristic of its *Rht-B1b* progenitor. Gel-blot analysis showed that *Rht-B1g* lacks C15-1-hybridizing DNA derived from chromosome 4B (the chromosome that carries *Rht-B1b*; Fig. 3b), indicating that *Rht-B1g* is a deletion mutation that abolishes *Rht-B1b* function.

The demonstration that, for multiple independent mutant alleles, a heritable change in phenotype is associated with a mutation in a candidate gene is conventionally used as proof that the candidate gene is indeed responsible for the phenotype being studied. Here we have shown that, for three independent mutant *d8* alleles, a heritable change in phenotype (dwarfism, reduced gibberellin response) is associated with a mutation in a candidate GAI-like gene. This shows

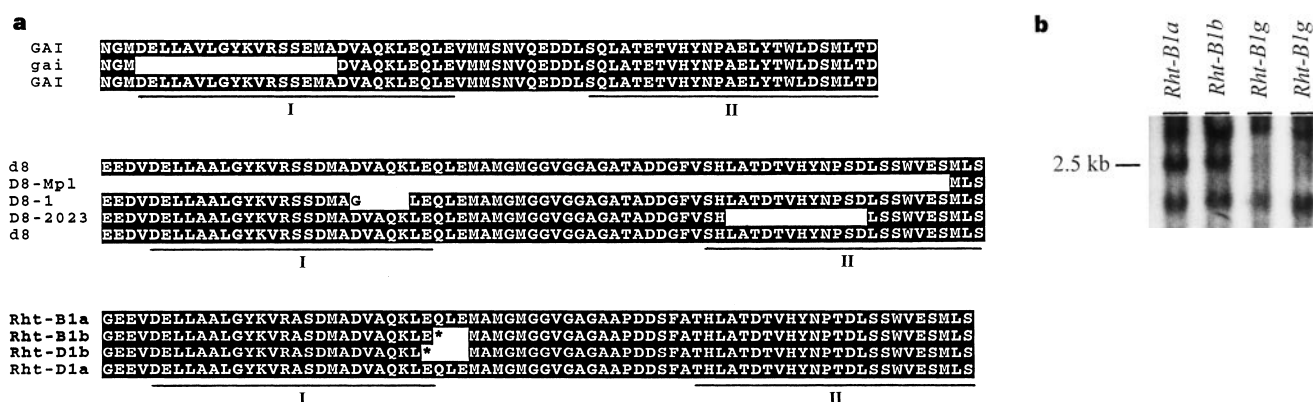


Figure 3 Dominant mutant alleles encode proteins with mutant N termini. **a**, N-terminal segments of predicted proteins encoded by mutant alleles *gai*, *D8-1*, *D8-2023*, *D8-Mpl*, *Rht-B1b* and *Rht-D1b* are compared with those of their respective wild-type alleles (*GAI*, *d8*, *Rht-B1a* and *Rht-D1a*). For each locus, the wild-type sequence is shown above and below the mutant sequence(s). Differences between wild-type and mutant sequences (deletions and substitutions) are highlighted in white, the position of translational stop codons is represented by an asterisk, and the previously identified highly conserved regions I and II (Fig. 2a) are shown. All mutations alter the N-terminal region of their encoded proteins, and affect regions I and/or II. *D8-2023* also carries a 6-bp deletion that removes one G and one A residue from 510GAGA513, and a nucleotide substitution that converts

T519 to A519 (with respect to *d8* sequence; Fig. 2a, and data not shown). Because these altered residues are poorly conserved in GAI/RGA/Rht-D1a/d8, these changes are considered not to be of phenotypic significance. In wheat, Q64 of *Rht-B1a* is equivalent to Q62 of *Rht-D1a*, owing to a difference of two amino-acid residues in a poorly conserved N-terminal region (see text; data not shown). *gai* was isolated following X-irradiation mutagenesis⁸; all other mutant alleles shown are of spontaneous origin^{4,6,7}. **b**, Gel-blot hybridization of C15-1 with *Bam*HI-digested DNA from *Rht-B1a* (var. Mercia), *Rht-B1b* (Mercia near-isogenic line) and two *Rht-B1g* homozygotes. A hybridizing 2.5-kb *Bam*HI fragment (assigned to chromosome 4B by nullisomic-tetrasomic analysis; data not shown) is missing in the *Rht-B1g* samples.



Figure 4 Basmati rice is dwarfed by a construct containing the *gai* ORF. **a**, Primary transformants were allowed to set seed (by self-pollination), and seedling gibberellin-response tests¹² were performed on the progeny. Results from two independent transformant families are shown. Each pair of seedlings consists of two seedlings from the same family: left, a segregant lacking the transgene and

displaying the classical elongation response to applied gibberellin; right, a segregant that contains the transgene and is relatively unresponsive to the applied gibberellin. **b**, Adult plant phenotypes. Right, tall plant lacking the transgene is a segregant from the same family as the dwarf plant (left) that contains the transgene.

that we have cloned maize *d8*. The protein encoded by *d8* is closely related to that encoded by the wheat *GAI*-like genes (*d8* shows 88% amino-acid identity with *Rht-B1a* and *Rht-D1a*), and these genes all map to the same region of the 'ancestral' cereal genome¹⁷. Thus it is reasonable to assume that maize *d8* and the wheat *GAI*-like genes are the same gene (are orthologues) in these two species. As a whole, our results show that each of five independent dominant mutant alleles (at *d8* and *Rht-1*) is associated with a mutation in this orthologous *GAI*-like gene, demonstrating that we have cloned *Rht-1* and *d8*. The deletion in *Rht-B1g* provides further confirmation that *Rht-B1* is an orthologue of *Arabidopsis GAI*, and that *Rht-B1b* is a mutant allele of this cloned gene.

Height reduction has been associated with yield increases and yield stability in a number of different crop species³. Dwarfing mutant alleles of *GAI*, *Rht-1* or *d8* can now be used directly to reduce the height of diverse crops. As a test of this, we introduced constructs expressing the *gai* protein into Basmati 370 rice (Fig. 4a, b). This rice is commonly grown in northern and north-western regions of the Indian subcontinent. Basmati 370 grain is popular because it is long and slender, is translucent white, cooks well and has a pleasant aroma. However, the plants are tall, with weak culms (stems), and are highly susceptible to damage by wind and rain. This damage causes considerable yield losses and a reduction in grain quality. Previous attempts (using conventional breeding methods) to reduce the height of Basmati 370 while retaining its good qualities were not successful owing to loss of the unique characters for which it is valued. In our experiments, seedling segregants carrying the *gai*-expressing construct exhibited reduced responses to gibberellin, whereas segregants lacking the transgene responded normally to gibberellin (Fig. 4a). Adult plants carrying the transgene were dwarfed with respect to control segregants lacking the transgene (Fig. 4b). It is now possible to insert a single, genetically dominant, potentially yield-enhancing, dwarfing gene into the genome of any transformable crop, without the need

for long-term conventional breeding programmes and with minimal disruption of genetic background.

Our results show that *Arabidopsis GAI*, wheat *Rht-1* and maize *d8* are functional orthologues. Gibberellin signalling appears to be very similar in monocotyledonous and dicotyledonous plants, and may involve the interaction of an SH2-like domain with a phosphorylated tyrosine residue. The mutations in the dominant dwarfing alleles of *D8* and *Rht-1*, like the mutation in the *gai* allele, affect the N-terminal region of the proteins that they encode. Previously, we proposed that *GAI* is a growth repressor whose action is opposed by gibberellin, and that *gai* is a mutant repressor that is relatively insensitive to the effects gibberellin^{6,9}. According to this view, our data show that a range of different N-terminal deletions and truncations convert *GAI/Rht-B1a/Rht-D1a/d8* into mutant repressors that are less affected by gibberellin than the normal protein. This confirms the importance of this N-terminal region for gibberellin signalling and is also consistent with the 'altered function' mode of dominance exhibited by the dominant mutant alleles of *GAI*, *Rht-1* and *d8*^{6,7,9,12}. Gibberellin elicits plant responses in a dose-dependent fashion¹⁵. The fact that different dominant mutant alleles of *Rht-1* and *d8* confer differing severities of dwarfism⁴⁻⁷ indicates that one of the functions of *GAI/RGA/Rht-B1a/Rht-D1a/d8* may be to modulate the gibberellin dose-response. Different amino-terminal deletions and truncations may differentially alter the magnitude of response to a given gibberellin dose, and the structure of this amino-terminal region may be key to the modulator function of *GAI/RGA/Rht-B1a/Rht-D1a/d8*. □

Methods

Molecular cloning, DNA gel-blot hybridization and DNA sequencing. We isolated wheat cDNA and genomic DNA and maize genomic DNA clones using low-stringency library screens²⁵. Wheat DNA gel-blot hybridizations were performed as described²⁶. Wheat genomic DNA clones were assigned to their chromosome of origin (4A, 4B or 4D) by identification of restriction fragments

previously assigned through DNA gel-blot analysis of nullisomic-tetrasomic lines. DNA sequencing was done using the Big Dye terminator cycle sequencing kit (Perkin Elmer). The entire coding sequence of each mutant gene (and of wild-type controls) was amplified from genomic DNA (using primers specific to *Rht-B1*, *Rht-D1* or *d8*, as appropriate) by PCR (GeneAmp XL PCR kit, Perkin Elmer). All wild-type and mutant *Rht-1* alleles were amplified from homozygous material. Amplification products were cloned into the pGEM-T Easy vector (Promega). For each gene, we determined DNA sequences from at least two independent amplifications, thus avoiding potential PCR-induced errors. Genetic analyses confirmed that the mutant *D8-1* and *Rht-D1b* sequences co-segregated with their respective mutant phenotypes. For *D8-1*, PCR analysis of the F₁ progeny of a *D8-1/d8* × *d8/d8* cross revealed five dwarf (*D8-1/d8*) plants that were heterozygous for the deletion mutation associated with *D8-1* (see text) and five tall (*d8/d8*) plants that did not carry this deletion. For *Rht-D1b*, the associated nucleotide substitution (see text) was found in three dwarf (*Rht-D1b/Rht-D1b*) but not in three tall (*Rht-D1a/Rht-D1a*) F₂ progeny of a *Rht-D1a/Rht-D1a* × *Rht-D1b/Rht-D1b* cross.

Isolation of *Rht-B1g*. We irradiated 3,000 wheat seeds (var. Highbury, homozygous for *Rht-B1b*) with 3.0 Gy fast-neutrons. *Rht-B1g* was identified as a tall, gibberellin-responsive¹² segregant in an M₂ family derived from self-pollination of an M₁ plant.

Rice transformants. We generated transgenic rice plants expressing the *Arabidopsis* *gai* protein by particle-gun-mediated transformation²⁷ using a construct in which the *gai* ORF was expressed under the control of the maize ubiquitin promoter. Presence of the *gai*-containing transgene was verified by PCR amplification⁹. The progeny (derived from self-pollination) of six independent primary transgenic plants were tested for segregation of the transgene and for gibberellin response¹². In all six families, the transgene and phenotype were perfectly co-segregated: all plants exhibiting a normal gibberellin response lacked a detectable transgene, and all plants exhibiting a reduced gibberellin response contained the transgene. Thus, the reduced gibberellin response phenotype is due to the transgene, and not to inactivation of genes resulting from insertion of the transgene into the rice genome, or to genetic variation generated by the transformation procedure *per se*. Control transformants containing the vector but lacking *gai* were not dwarfed (data not shown).

Illustrations. The amino-acid sequence alignments in Fig. 2a were done using software from the Wisconsin Package (Genetics Computer Group) with default parameters. Alignments in Fig. 2b were made by eye.

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Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1

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The underlying causes of obesity are poorly understood but probably involve complex interactions between many neurotransmitter and neuropeptide systems involved in the regulation of food intake and energy balance. Three pieces of evidence indicate that the neuropeptide melanin-concentrating hormone (MCH) is an important component of this system. First, MCH stimulates feeding when injected directly into rat brains^{1,2}; second, the messenger RNA for the MCH precursor is upregulated in the hypothalamus of genetically obese mice and in fasted animals¹; and third, mice lacking MCH eat less and are lean³. MCH antagonists might, therefore, provide a treatment for obesity. However, the development of such molecules has been hampered because the identity of the MCH receptor has been unknown until now. Here we show that the 353-amino-acid human orphan G-protein-coupled receptor SLC-1 (ref. 4) expressed in HEK293 cells binds MCH with sub-nanomolar affinity, and is stimulated by MCH to mobilize intracellular Ca²⁺ and reduce forskolin-elevated cyclic AMP levels. We also show that SLC-1 messenger RNA and protein is expressed in the ventromedial and dorsomedial nuclei of the hypothalamus, consistent with a role for SLC-1 in mediating the effects of MCH on feeding.

We cloned a 353-amino-acid orphan G-protein-coupled receptor from a human fetal brain complementary DNA library that was identical to that subsequently described as the 'corrected' sequence for human SLC-1, or GPR24 (ref. 4). SLC-1 contains a number of features typical of members of the G-protein-coupled receptor superfamily, including seven putative transmembrane domains⁵, a DRY motif at the boundary of transmembrane domain 3 and the second intracellular loop^{3,6}, consensus sites for asparagine-linked glycosylation at the amino terminus⁴ and other features⁴. Southern blot analysis using the SLC-1 coding region under low-stringency hybridization conditions revealed only one hybridization band in genomic DNA samples from each of human, monkey, rabbit, cow, rat, mouse and dog. This indicates that there is no SLC-1 subtype that closely resembles SLC-1 at the DNA level, and supports similar conclusions previously reported⁵, but does not eliminate the possible existence of SLC-1 subtypes with low sequence homology.

We used a 'reverse pharmacology' approach⁷ to identify the natural cognate ligand for SLC-1. The receptor was expressed in HEK293 and screened against a large library of known bioactive substances, including over 500 naturally occurring or putative neuropeptides. In this screen, MCH (human, rat and mouse are identical) was the only substance to produce a robust, dose-dependent ($EC_{50} = 3.72$ nM), transient elevation of intracellular calcium in HEK293 cells transiently transfected with SLC-1. In control experiments, cells transiently transfected with empty vector (Fig. 1) or OX_1 receptors⁸ did not respond to MCH. OX_1 -receptor-mediated orexin-A responses were similar in size and nature to SLC-1-mediated MCH responses (Fig. 1).

HEK293 cells stably transfected with SLC-1 also responded to MCH with a robust, dose-dependent, transient elevation of intracellular calcium with an EC_{50} of 7.91 nM (Fig. 2a and Table 1). The cells also responded to MCH with a dose-dependent inhibition of forskolin-elevated intracellular cAMP (Fig. 2b and Table 1) that was abolished by overnight pre-incubation with pertussis toxin (25 ng ml⁻¹), indicating that the response was mediated by a G-protein α -subunit of the $G_{i/o}$ family (data not shown). Also, radioligand-binding studies with membranes prepared from these cells showed saturable specific binding of [¹²⁵I] MCH with sub-nanomolar affinity ($K_d = 0.214 \pm 0.017$ nM, $n = 3$), and with a B_{max} of 181 ± 20 fmol mg⁻¹ protein (Fig. 3a). No significant specific binding was detected in wild-type cells. Saturation isotherms did not fit a two-site model significantly better than a simple one-site model. In competition binding experiments using 0.2 nM [¹²⁵I] MCH, specific binding (representing 68% of the total binding) was strongly displaced by human MCH (Fig. 3b and Table 1), and 94% of the specific binding was inhibited by 100 μ M guanosine-5'-O-(3-thiotriphosphate), indicating that binding is largely to the high-affinity state of the receptor under these conditions.

In both functional assays, and in the binding assay, only three

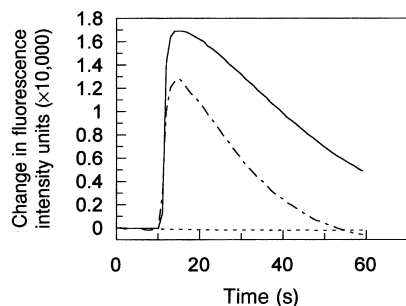


Figure 1 MCH activates SLC-1 transiently transfected into HEK293 cells. Cells transfected with SLC-1 (continuous line), but not those transfected with empty vector (dotted line), responded to 10 nM MCH, added at the 10 s time-point, with a robust, transient elevation of intracellular calcium. As a positive control, cells transfected with the orexin receptor, OX_1 , responded to orexin-A with a similar response profile in this system (dot-dashed line).

peptides showed high potency: human MCH, salmon MCH and a synthetic analogue of MCH, [Phe¹³, Tyr¹⁹]-MCH (Figs 2a, b and 3b and Table 1). A variant form of MCH, the putative product of a second, variant form of the MCH gene⁹, differing from authentic human MCH in four amino acids (see Methods), was about 100–1,000 times less potent than authentic MCH (Figs 2a, b and 3b and Table 1). Because of the low potency of variant-MCH at SLC-1, and evidence that such a peptide is unlikely to be naturally expressed¹⁰, we suggest that variant-MCH is unlikely to be a natural ligand for SLC-1.

To confirm the specificity of SLC-1 activation by MCH we tested a number of other peptides. Three putative neuropeptides derived from the authentic MCH precursor (neuropeptide-EI and neuropeptide-GE (ref. 11) and MCH-gene-overprinted polypeptide-14 (ref. 12)), and an equivalent putative peptide from the variant MCH precursor gene (variant-neuropeptide-EI (ref. 9)), were inactive as agonists in both functional assays, did not inhibit radioligand binding and did not inhibit MCH-induced calcium mobilization in HEK293 cells stably expressing SLC-1 when tested at concentrations up to 10 μ M (data not shown). We also tested somatostatin-14, as SLC-1 is most closely related to somatostatin receptors⁵, and found it to be inactive.

Evidence for cell lines that express endogenous MCH receptors has been reported. [¹²⁵I][Phe¹³, Tyr¹⁹]-MCH bound specifically and with high affinity to sites on SVK14 keratinocytes¹³ and mouse melanoma cells¹⁴ ($K_d = 0.7$ and 0.118 nM, respectively), and the binding was weakly displaced by a number of atrial, brain and C-type natriuretic peptides (K_i values between 116 and 365 nM). However, rat atrial natriuretic peptide (ANP)(1–28), rat ANP(3–28), human ANP(3–28), human C-type natriuretic peptide-22 and human brain natriuretic peptide-32 had no detectable affinity for, or functional activity at, SLC-1 when tested at up to 10 μ M. In addition, the affinity of MCH for the site on these cells is over 275

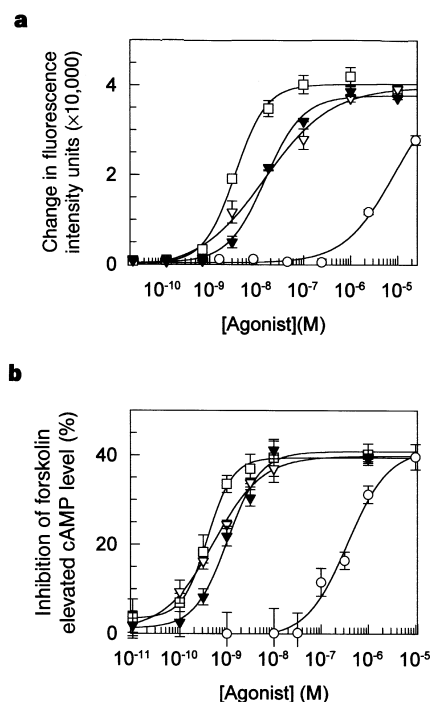


Figure 2 MCH and related peptides activate SLC-1 in two different functional assays. HEK293 cells stably transfected with SLC-1 responded in a dose-dependent fashion to MCH peptides, with transient elevations of intracellular Ca^{2+} (a) and an inhibition of forskolin-elevated levels of cAMP (b). Peptides shown; human MCH (open squares), salmon MCH (open triangles), [Phe¹³, Tyr¹⁹]-MCH (filled triangles) and variant-MCH (open circles). Data are from a single representative experiment. Each point determined in triplicate and given as a mean $\pm$ s.e.m.

Table 1 Functional activity and binding affinities of MCH-related peptides at SLC-1 stably expressed in HEK293 cells

Peptide	Intracellular calcium mobilization; EC ₅₀ , n = 3	Inhibition of forskolin elevated intracellular cAMP; EC ₅₀ , n = 5	Inhibition of [¹²⁵ I]MCH binding; K _i , n = 3
Human MCH	7.91 ± 3.8	0.276 ± 0.047	0.043 ± 0.009
Salmon MCH	26.6 ± 11.8	0.518 ± 0.074	0.386 ± 0.037
[Phe ¹³ , Tyr ¹⁹]-MCH	26.3 ± 13.4	0.668 ± 0.080	0.115 ± 0.017
Variant-MCH	>1000	291.8 ± 24.4	294.7 ± 34.8

Potencies (nM) are given as means of the EC₅₀ and K_i values obtained from 3–5 separate experiments (± s.e.m.).

times lower than at SLC-1 (K_i of 65–93 nM¹³ and 12–120 nM¹⁴ vs 0.043 nM (Table 1)). These pharmacological differences may arise from the expression of SLC-1 in different hosts, or from subtypes of SLC-1 in these cells. It will therefore be of interest to investigate the expression of SLC-1-like sequences in these cells.

MCH can act as a functional antagonist of α -melanocyte stimulating hormone (α -MSH) in a diverse range of animal species and physiological roles. In teleost fish, where MCH was first discovered¹⁵, MCH lightens skin pigment cells and α -MSH darkens them. In mammals, the effects of these peptides on skin pigmentation have been lost, but their mutually antagonistic relationship has been maintained in other physiological roles such as feeding. Thus, in mammals, MCH is orexigenic and α -MSH is anorexigenic^{16,17}. Here, α -MSH was tested at up to 10 μ M concentration and no agonistic or antagonistic interaction with SLC-1 was observed. Together with the observation that MCH cannot displace α -MSH from melanocortin receptors¹⁶, these results support the idea that the functional, mutually antagonistic effects of α -MSH and MCH are mediated by their interaction at separate receptors. It is tempting to speculate that the mechanism of this mutual antagonism may in part reside in the opposite intracellular signalling pathways activated by these peptides (to raise and lower intracellular cAMP

levels, respectively). Certainly, our observations are consistent with the general observation that neuropeptides that inhibit food intake (for example, α -MSH, corticotropin-releasing hormone, glucagon-like peptide-1 and cholecystokinin) all act by increasing protein kinase A signalling, whereas peptides that potently increase food intake (for example, neuropeptide Y and agouti-related protein) appear to act, in general, by reducing intracellular protein kinase A signalling.

Using a combination of *in situ* hybridization immunohistochemical techniques we investigated the distribution of SLC-1 in rat brain. Our *in situ* hybridization data agreed with the mRNA distribution of SLC-1 described previously⁵, confirming that SLC-1 is widely and strongly expressed in the rat brain. There were clear mRNA signals in the olfactory tubercle, cerebral cortex (frontal, piriform, somatosensory and cingulate), substantia nigra, basal forebrain, CA1, CA2 and CA3 fields of the hippocampus, amygdala, and various other nuclei in the hypothalamus, thalamus, midbrain and hindbrain. There were also strong signals in two nuclei of the hypothalamus not described in the original studies; the ventromedial (VMH) and dorsomedial (DMH) nuclei (Fig. 4), areas widely recognized as being involved in feeding behaviour. To confirm the expression of SLC-1 in these nuclei we raised a polyclonal antiserum directed against the carboxy-terminal sixteen amino-acid residues of rat and human SLC-1. Immunocytochemistry using an affinity-purified antiserum produced a clear membrane-related signal on HEK293 cells expressing SLC-1, but not on wild-type cells (Fig. 5a, b). In addition, pre-incubation of affinity-purified antiserum with the synthetic peptide (1 μ M) used as antigen markedly reduced the signal in SLC-1 cells (Fig. 5c),

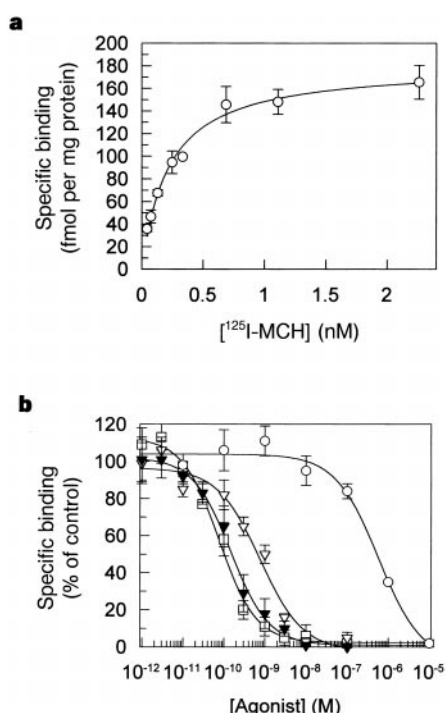


Figure 3 MCH peptides bind with high affinity to membranes prepared from HEK293 cells stably transfected with SLC-1. **a**, Saturation isotherm for binding of the radioligand [¹²⁵I]MCH. **b**, Inhibition of [¹²⁵I]MCH binding by MCH peptides. Peptides shown; human MCH (open squares), salmon MCH (open triangles), [Phe¹³, Tyr¹⁹]-MCH (filled triangles) and variant-MCH (open circles). Data are means ± s.e.m. from three individual experiments.

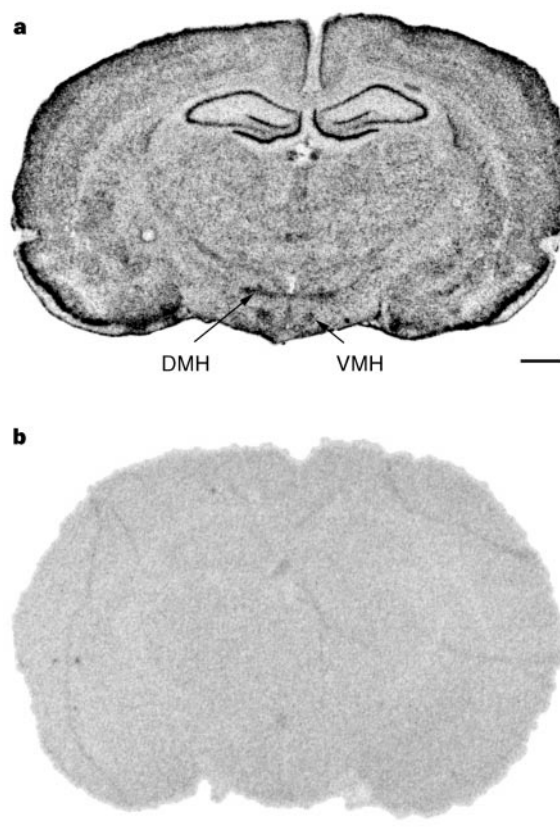


Figure 4 SLC-1 mRNA is present in the ventromedial and dorsomedial nuclei of the hypothalamus, areas implicated in feeding behaviour. **a**, Autoradiogram of a coronal section through the rat brain showing an *in situ* hybridization signal obtained using an oligonucleotide antisense probe specific for SLC-1. Note the dense labelling in the dorsomedial (DMH) and ventromedial (VMH) nuclei of the hypothalamus. **b**, Control section with 100-fold excess of unlabelled probe. Scale bar: 1 mm.

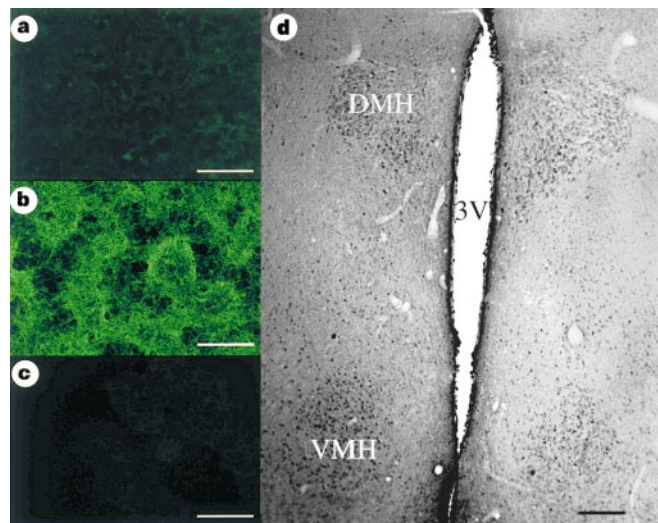


Figure 5 SLC-1 protein is present in the ventromedial and dorsomedial nuclei of the hypothalamus (demonstrated using an affinity-purified antiserum directed to the C-terminal tail of SLC-1). Immunocytochemistry was used to demonstrate the specificity of the antiserum for SLC-1. A clear membrane-related signal was absent from wild-type cells (**a**), but present on SLC-1-transfected HEK293 cells (**b**). Also, pre-incubation of affinity-purified antiserum with the synthetic peptide used as antigen (1 μ M) resulted in a marked loss of signal from SLC-1-transfected HEK293 cells (**c**). **d**, Immunohistochemical distribution of SLC-1-like antigenicity in a coronal section through the hypothalamus of a rat. Note the strong immunostaining of cell bodies in the DMH and VMH. 3V, third ventricle. Scale bars: **a–c**, 225 μ m; **d**, 200 μ m.

confirming the specificity of the antiserum. Using this antibody, we found immunostained cell bodies in the VMH and DMH (Fig. 5d). The correspondence of mRNA and protein expression patterns show that SLC-1 is localized in these nuclei, and indicate that SLC-1 may mediate the effects of MCH on feeding.

During our attempts to clone SLC-1 we cloned a splice variant form of this receptor, identical to that subsequently reported⁵, which is 49 amino acids longer and lacks three putative N-linked glycosylation sites present in the 'corrected' sequence⁴. HEK293 cells stably and transiently transfected with this splice variant of SLC-1 (ref. 5) responded weakly to high concentrations of MCH, giving only a small transient increase in calcium. Furthermore, we found no specific radioligand binding in membranes prepared from these cells. Immunocytochemistry of these cells, using the antiserum described above, did not produce staining significantly different to that of wild-type cells (data not shown), indicating that this splice variant may be expressed only at very low levels in these cells. These data are consistent with this splice variant of SLC-1 being a non-physiological, inappropriately spliced form of the receptor⁴. We are currently developing splice-variant-specific antisera to investigate whether this apparently aberrant splice variant is expressed in tissues.

The widespread distribution of MCH-containing neurons¹⁸ and SLC-1 (ref. 5) in the mammalian central nervous system, together with our data, indicates that the SLC-1-mediated effects of MCH may be involved in a wide variety of brain functions and behaviour, including feeding, and that antagonists of SLC-1 may provide a useful therapy for obesity. □

Methods

Isolation of human and mouse SLC-1 cDNAs. The coding portion of the human angiotensin II type 1 receptor cDNA¹⁹ was ³²P-radiolabelled and used to screen a human frontal cortex phage cDNA library (Stratagene) as described²⁰. Positive clones were plaque purified, and the inserts were manually sequenced (Sequenase, U.S.B. Corp.). One cDNA, encoding a 402-amino-acid protein, was identical to SLC-1 as reported⁵. An approximately 1,200-base pair (bp)

coding region from this cDNA was ³²P-radiolabelled and used to screen a mouse brain phage cDNA library as above. One clone, designated mSLC-1, contained a 2,135-bp insert encoding a 353-amino-acid protein with 91% identity at the amino-acid level to SLC-1; however, the homology between the N termini diverged and they were not the same length. Oligonucleotides corresponding to the 5'-most region conserved between SLC-1 and mSLC-1 were used, together with template human fetal brain cDNA (Gibco BRL), to obtain by 5' RACE another form of SLC-1 that is identical in length to and shares 96% identity with mSLC-1, and is 100% identical to a 'corrected' human SLC-1 described subsequently⁴. Oligonucleotides corresponding to the N and C termini of the 'corrected' human SLC-1 were used to amplify by PCR a cDNA encoding the complete sequence for this receptor. Southern blot analysis was carried out with ZOOBlot (Clontech Lab) using the SLC-1 receptor coding region as a probe under low-stringency hybridization conditions, as recommended by the manufacturer.

Receptor expression. Both splice variants of SLC-1 were subcloned into the mammalian expression vector pCDN (ref. 21) and transiently transfected into HEK293 cells using Lipofectamine Plus (Life Technology), according to the manufacturer's instructions. Stable clonal cell lines were produced by serial dilution into selective media containing 400 μ g ml⁻¹ G418, and screened by MCH-induced calcium mobilization.

Peptides. Variant-MCH (DFDTLSCMLGRVYQSCWQV), which differs from authentic MCH (DFDMLRCMLGRVYRCPWQV) by four residues at positions 4, 6, 14 and 15, and variant-neuropeptide-EI (ETGDENSAKFPV-amide)⁹ were both synthesized by Fmoc solid-phase peptide synthesis and oxidized with DMSO in TFA²². All other peptides were obtained from Bachem UK, Sigma or Phoenix Pharmaceuticals.

Calcium-mobilization assays. HEK293 cells were seeded (50,000 cells per well) into poly-D-lysine-coated 96-well black-wall, clear-bottom microtitre plates (Becton Dickinson) 24 h before assay. Cells were loaded for 1 h with 1 μ M Fluo-4-AM fluorescent indicator dye (Molecular Probes) in assay buffer (Hanks Balanced Salts Solution (HBSS), 10 mM HEPES, 200 μ M Ca²⁺, 0.1%BSA, 2.5 mM probenecid), washed three times with assay buffer, then returned to the incubator for 10 min before assay on a fluorimetric imaging plate reader (Molecular Devices). We used the maximum change in fluorescence over baseline to determine agonist response. For antagonist studies, we added test substances 30 min before agonist addition. Dose-response curve data were fitted to a four-parameter logistic equation using GraFit (Erithacus Software Limited).

cAMP assays. Intracellular cAMP was determined with flashplates (SMP004, New England Nuclear). Briefly, cells were dispensed into 96-well flashplates (50,000 cells per well) at 37°C and incubated with 0.5 mM isobutylmethyl-xanthine for 15 min before test peptides and forskolin (30 μ M) were added simultaneously to a final volume of 100 μ l. After 15 min the incubation was terminated and plates processed according to the manufacturer's instructions. Plates were counted on a TopCount (Packard). Dose-response curve data were fitted to a four-parameter logistic equation using GraFit (Erithacus Software Limited).

Radioligand binding assays. Membrane preparation and radioligand binding assays were performed essentially as described²³ with minor modifications. Briefly, competition experiments were performed using 0.2 nM [¹²⁵I] MCH (provided by New England Nuclear), with a specific activity of 2,200 Ci mmol⁻¹. Non-specific binding was defined using 1 μ M MCH. Assays were initiated by the addition of membranes (2–4 μ g of protein) and terminated after 1 h at 22°C by filtration through Whatman GF/C glass-fibre filters, pre-soaked in 0.3% non-fat dry milk. We analysed data using GraphPad Prism (San Diego, CA).

In situ hybridization. Methods were as described²⁴. Rat SLC-1 sequence, ³⁵S-radiolabelled oligoprobes were found to be unique to rat SLC-1 using the BLAST program. Various oligonucleotides with non-overlapping sequences were used independently and provided similar patterns of distribution, although with different intensities of labelling. The oligonucleotide 5'-ATA CTA CAG CGC ATG ACG TCT TCG GTC GCC CC-3' was used in subsequent experiments. Control experiments included the use of sense-sequence oligoprobes, a 100-fold excess of non-labelled antisense oligoprobes and RNase-A pretreated sections.

Immunohistochemistry. A rabbit polyclonal antiserum against a C-terminal peptide of the SLC-1 sequence (H-SNAQTADERTESKGT-OH) was raised

and affinity-purified as described²⁵. Immunocytochemical fluorescence was carried out as described^{24,25}. Controls included the omission of the primary antisera, the use of pre-immune rabbit serum and the pre-incubation of the affinity-purified antiserum with the synthetic peptide (1 μ M) used as antigen.

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Molecular characterization of the melanin-concentrating-hormone receptor

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Orphan G-protein-coupled receptors (GPCRs) are cloned proteins with structural characteristics common to the GPCRs but that bind unidentified ligands. Orphan GPCRs have been used as targets to identify novel transmitter molecules¹. Here we describe the isolation from brain extracts and the characterization of the natural ligand of a particular orphan GPCR (SLC-1) that is

sequentially homologous to the somatostatin receptors^{2,3}. We show that the natural ligand of this receptor is the neuropeptide melanin-concentrating hormone (MCH)⁴. MCH is a cyclic peptide that regulates a variety of functions in the mammalian brain, in particular feeding behaviour^{5,6}. We demonstrate that nanomolar concentrations of MCH strongly activate SLC-1-related pathways through $G\alpha_i$ and/or $G\alpha_q$ proteins. We have analysed the tissue localization of the MCH receptor and find that it is expressed in several brain regions, in particular those involved in olfactory learning and reinforcement mechanisms, indicating that therapies targeting the MCH receptor should act on the neuronal regulation of food consumption.

The application of homology screening approaches has led to the identification of many GPCRs, some of which, the so-called orphan

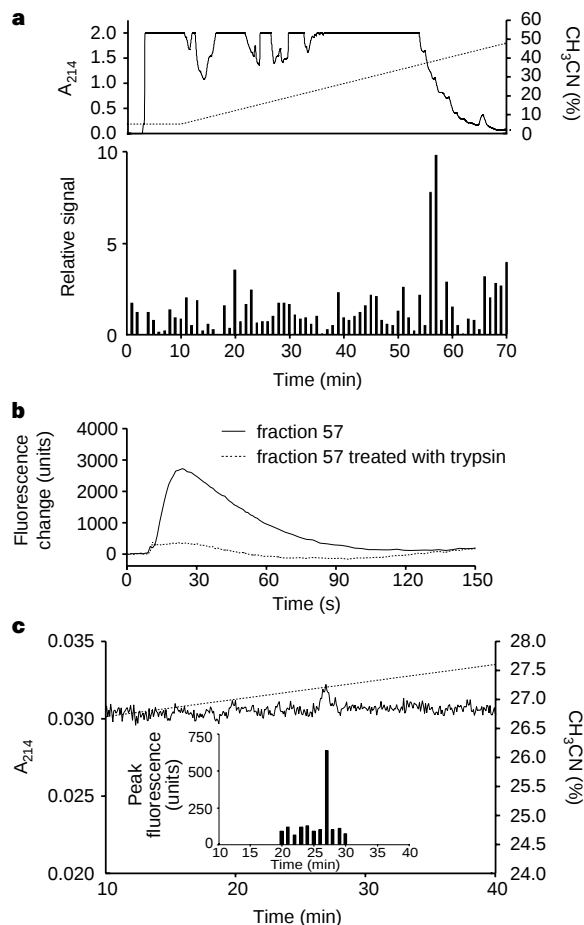


Figure 1 Purification of SLC-1 endogenous ligand from rat brain extracts. CHO cells were transiently transfected with SLC-1 and the $G\alpha_{q/13}$ chimera. Increases in $[Ca^{2+}]_i$ induced by the fractions eluted from the HPLC were monitored using the FLIPR detection system. **a**, C18 reverse-phase HPLC (PrePAK-Delta-Pak 25 $\times$ 100 mm) elution profile of the extract. Elution was performed with a linear gradient of CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 1 ml min⁻¹. Relative $[Ca^{2+}]_i$ signal is expressed as a ratio of the increment obtained by SLC-1- $G\alpha_{q/13}$ transfected cells versus orphanin FQ-receptor- $G\alpha_{q/13}$ -transfected cells. Active fractions 56 and 57 were combined for further purification. **b**, Kinetics of the $[Ca^{2+}]_i$ changes evoked by fraction 57. The fraction was treated with trypsin attached to agarose beads (20 mU) for 3 h at 37°C and the reaction was terminated by removing the beads by centrifugation. Intracellular calcium fluxes were monitored in the FLIPR detection system. **c**, Final purification of the active compound by Sephasil C8 SC2.1/10 using the SMART system. Elution was performed with a linear gradient of CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 0.1 ml min⁻¹. Inset the peak increments in $[Ca^{2+}]_i$ induced by designated HPLC fractions. Solid line, absorption at 214 nm (A_{214}); dotted line, percentage of CH_3CN .

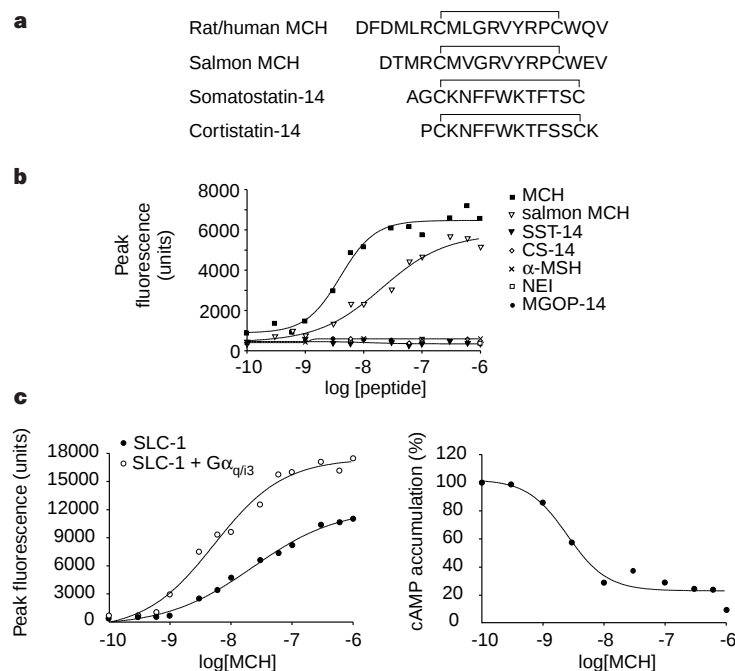


Figure 2 Specificity of the interaction between MCH and SLC-1. **a**, Alignment of rat/human MCH sequence with salmon MCH, somatostatin-14 and cortistatin-14. **b**, Dose-response curves of the $[Ca^{2+}]_i$ changes evoked in CHO cells transfected with SLC-1 and $G\alpha_{q/i3}$ by MCH, salmon MCH, somatostatin-14 (SST-14), cortistatin-14 (CS-14), α -MSH, NEI and MGOP-14. Somatostatin analogue RC-160, cortistatin-29, and MGOP-27 did not induce Ca^{2+} influx in the SLC-1- $G\alpha_{q/i3}$ co-transfection. All incubations were done at least three times. Representative results are shown. **c**, Diversity of the MCH-induced SLC-1 signalling in CHO cells.

Left, comparison of the dose-response curves for changes in $[Ca^{2+}]_i$ induced by SLC-1 alone or SLC-1 co-expressed with $G\alpha_{q/i3}$, showing that SLC-1 can induce $[Ca^{2+}]_i$. Right, inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with SLC-1 alone, showing that SLC-1 can also induce inhibition of adenyl cyclase. Data were normalized to the amounts of cAMP in forskolin-stimulated cells (100%). All incubations were done in triplicate and representative data are shown.

GPCRs¹, recognize unidentified signalling molecules. Many of these orphan GPCRs are likely to be receptors for new neurotransmitters or neuropeptides and thus represent useful resources for drug discovery¹. In 1995, an orphan GPCR was used as a target to identify and isolate a novel neuropeptide^{7,8}. Since then, three more neuropeptides have been identified using the orphan-receptor strategy^{9,10,11}. The orphan GPCR SLC-1 (also called GPR24)^{2,3}, exhibits about 40% amino-acid identity to five known human somatostatin receptors ($G\alpha_i$ -linked). On the assumption that SLC-1 may also bind a peptidic ligand and couple to $G\alpha_i$ proteins, we collected different tissues that express SLC-1 and processed them for peptide extraction using different protocols^{7,9,10}. Each chromatographic fraction was tested for its ability to induce a decrease in cyclic AMP in forskolin-stimulated, SLC-1-transfected Chinese hamster ovary (CHO) cells. None of the fractions showed a response that could be reproducibly followed over several purification steps. We therefore developed a new method to monitor SLC-1 activity by recording calcium influxes. Our goal was to force SLC-1 to couple to a $G\alpha_q$ protein. Because the five carboxy-terminal residues of $G\alpha$ are sufficient for receptor contact, whereas the rest of the subunit interacts with the effector molecule, a $G\alpha_{q/i3}$ chimaera designed to drive SLC-1 to $G\alpha_q$ activation was constructed that contained the five C-terminal residues of $G\alpha_{i3}$ and retained the rest of the $G\alpha_q$ sequence^{12,13}. Using total rat brain extracts, we identified two consecutive high-performance liquid chromatography (HPLC) fractions that elicited a robust increase in cytoplasmic calcium in CHO cells co-transfected with SLC-1 and the $G\alpha_{q/i3}$ chimaera complementary DNAs (Fig. 1a, b). The activities detected in these two fractions (fractions 56 and 57) were specific to the SLC-1- $G\alpha_{q/i3}$ system, as they did not induce calcium influx in cells transfected with ORL-1- $G\alpha_{q/i3}$ (Fig. 1a). The same fractions elicited an increase in calcium levels when we co-transfected SLC-1- $G\alpha_{q/i3}$ into human embryonic kidney (HEK) 293-T cells. This effect appeared to be

mediated by a peptide, as treatment with trypsin abolished the activity (Fig. 1b).

Fractions 56 and 57 were pooled and further purified by six more chromatographic steps (Fig. 1c, and see Methods). The final material was subjected to structural analysis by MALDI mass spectrometry and Edman degradation. Amino-acid sequence analysis revealed an amino-terminal sequence in which three of the first five residues were identical to those of MCH¹⁴. As synthetic rat MCH was shown to behave identically to our purified peptide in retention time (by reverse-phase HPLC) and in molecular size (by mass data), we inferred that our peptide is MCH. Final yields of MCH were ~ 25 pmol kg⁻¹ for rat brain (wet weight). Note that the longer human SLC-1 isoform² was inactive in our assay system.

Synthetic rat MCH induced a dose-dependent transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in transiently transfected rat SLC-1- $G\alpha_{q/i3}$ cells (Fig. 2b), but failed to induce detectable $[Ca^{2+}]_i$ changes in mock-transfected CHO cells (data not shown). The MCH concentration required to induce a half-maximum response (EC_{50}) was 4.8 ± 0.5 nM, confirming that MCH is an endogenous ligand for SLC-1. Salmon MCH⁴, which has a high degree of homology to rat MCH in its central and C-terminal portions (Fig. 2a), activates SLC-1 with an EC_{50} of 18.6 ± 2.3 nM. Because SLC-1 shares sequence similarities with the somatostatin receptors and because somatostatin has a circular topology similar to that of MCH (Fig. 2a), somatostatin-14 and the somatostatin analogue RC-160 were tested on SLC-1- $G\alpha_{q/i3}$ -transfected cells. They did not induce an increase in $[Ca^{2+}]_i$ in our cell assay (Fig. 2b). Cortistatin-14 or -29 (ref. 15), which are structurally related to somatostatin (Fig. 2a), also failed to induce detectable $[Ca^{2+}]_i$ changes (Fig. 2b). Moreover, somatostatin-14 and cortistatin-14 or -29 did not antagonize the MCH-activated SLC-1 response (data not shown). MCH-gene-related peptides (MCH-precursor-derived peptide, NEI; MCH-gene-overprinted-polypeptide,

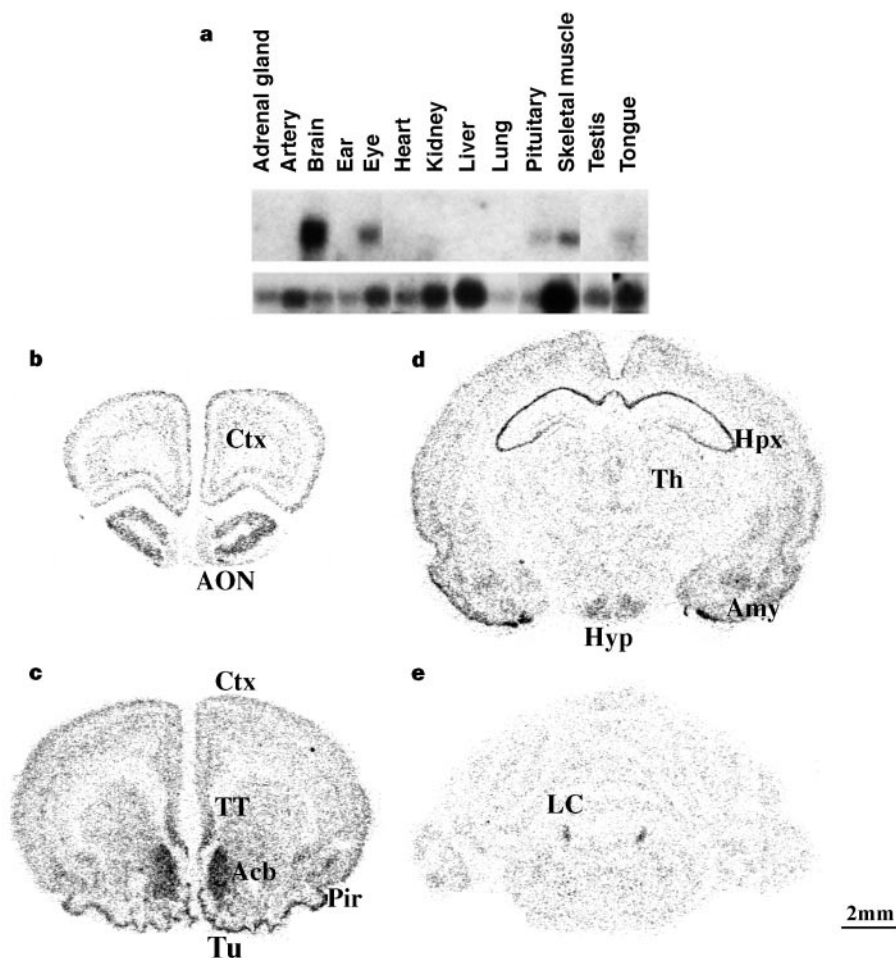


Figure 3 Regional distribution of SLC-1 mRNA. **a**, Northern blot analysis. 3 μ g per lane of poly(A)⁺ RNA from indicated rat tissues was analysed by northern blotting using SLC-1 cDNA probes (top). Loading was verified by hybridization to a G3PDH control probe (bottom). **b–e**, Localization of SLC-1 transcripts in rat brain sections by *in situ* hybridization using a cRNA probe (see Methods). Ctx: cortex;

AON: anterior olfactory nucleus; TT: taenia tecta; Tu: olfactory tubercle; Acb: nucleus accumbens; Pir: piriform cortex; Hpx: hippocampus; Th: thalamus; Hyp: hypothalamus; Amy: amygdala; LC: locus coeruleus. Control hybridization with a sense strand cRNA produced no specific signal (data not shown).

MGOP-14 or -27)^{16,17}, and α -melanotropin (MSH) also failed to induce a transient increase in $[Ca^{2+}]_i$ in transfected SLC-1- $G\alpha_{q/13}$ cells (Fig. 2b). Thus, SLC-1 is a specific receptor for MCH, and consequently the other peptides derived from the MCH gene, if they are bioactive, must bind to different receptors.

MCH and α -MSH have opposite effects on skin colour in teleost fishes¹⁸ and exert antagonistic influences on a variety of physiological functions including feeding behaviour^{19–22}. MCH also antagonizes the effect of NEI on grooming and locomotor activities²¹. When tested in our cell system, at concentrations of 1 nM–1 μ M, neither α -MSH nor NEI could block calcium mobilization induced by MCH (data not shown). As MCH is not recognized by the melanocortin receptors²², and as we now demonstrate that α -MSH does not bind the MCH receptor, we infer that the physiological antagonism of these two molecules results from the convergence of signalling pathways activated by distinct receptors.

To determine the signalling pathways of SLC-1, we established a CHO cell line that stably expressed SLC-1. In these cells, MCH induced robust increases in $[Ca^{2+}]_i$, with an EC_{50} of 18.2 ± 4.6 nM (Fig. 2c, left). When $G\alpha_{q/13}$ was transiently transfected into these stable SLC-1-expressing cells, the EC_{50} for MCH on $[Ca^{2+}]_i$ release was 4.2 ± 0.8 nM (Fig. 2c, left), the same value as was found for the SLC-1- $G\alpha_{q/13}$ transient co-transfection (Fig. 2b). Furthermore, in stably transfected SLC-1 cells, MCH potently inhibited forskolin-stimulated cAMP accumulation (Fig. 2c, right). The EC_{50} (4.1 ± 1.7 nM) for the cAMP assay is similar to that found when the $G\alpha_{q/13}$ chimera is expressed. Together, these data indicate that SLC-1 couples not only to

G_i but also to G_q , albeit with a lower affinity. The fact that SLC-1 can couple to different G proteins indicates that it may activate different second messenger responses in distinct cellular environments.

Northern blot analyses of adult rat tissues showed that the 2-kilobase (kb) SLC-1 messenger RNA is detected at a high level in the brain, in moderate amounts in the eye and skeletal muscle, and in small amounts in tongue and pituitary (Fig. 3a). A possible role for MCH in the eye, skeletal muscle and tongue has not been previously investigated, to our knowledge. The existence of SLC-1 in the pituitary supports a neuroendocrine role for MCH^{22,23}. To localize SLC-1 expression more precisely within the central nervous system, we carried out *in situ* hybridization using an antisense RNA probe on rat brain sections (Fig. 3b–e). There was extensive SLC-1 expression in the hippocampal formation, olfactory regions and the medial nucleus accumbens (Fig. 3b–d). This distribution corresponds to the monosynaptic connections that MCH neurons make with several areas in the brain involved in integrating inputs related to taste and olfaction^{24,25}. SLC-1 localization in these areas reveals a possible role for MCH in olfactory learning and reinforcement mechanisms, which are fundamental processes in the regulation of feeding behaviour. The presence of SLC-1 in parts of the hypothalamus that regulate feeding and metabolism, such as the ventromedial nucleus (VMH) (Fig. 3d), further supports this hypothesis. Moderate expression of SLC-1 mRNA was found in the substantia nigra, ventral tegmental area and in the amygdala (Fig. 3c, d), indicating that MCH may modulate the dopaminergic system. We also detect moderate expression in the locus coeruleus

(Fig. 3e), which indicates that MCH may participate in the control of various noradrenaline-modulated responses including vigilance, attention, memory and sleep.

MCH is a cyclic 19-amino-acid polypeptide that is synthesized mainly in the lateral hypothalamus and zona incerta^{24,25}. It is important for integrating and influencing the complex physiology underlying feeding^{5,6,22}. Central administration of MCH promotes feeding^{5,22} and MCH mRNA increases as a result of starvation and leptin deficiency⁵. Mice with no MCH have reduced body weight and leanness due to hypophagia⁶. As both leptin and pro-opiomelanocortin mRNA are reduced in these mice, MCH is thought to regulate feeding and energy balance by acting downstream of leptin and the melanocortin system. Functional relationships may exist between the MCH, leptin and MSH systems, as their receptors are co-expressed in several areas, in particular in the hypothalamus^{26,27}, and possibly in the same neurons. Our results will allow further study of these interactions by focusing on molecular analyses at the receptor levels.

In conclusion, we have identified MCH as a natural ligand for the orphan GPCR SLC-1. To do this, we developed an assay that can monitor orphan GPCRs linked to a variety of G-proteins. This study demonstrates the use of this strategy to identify the natural ligand of an orphan GPCR and indicates the wide applicability of this approach. □

Methods

Cell transfection. Full-length rat SLC-1 cDNA was cloned by PCR using specific oligonucleotides³ from a rat brain Marathon cDNA library (Clontech). The resulting 1.1-kb PCR product was subcloned into a pcDNA 3.1 (+) expression vector and sequenced. We constructed Gα_q chimaeras containing Gα_q residues 1–354 and the C-terminal five residues (ECGLY) of a known Gα_q (ref. 12), designed Gα_{q/13}. Construction of Gα_{q/13} chimaeras using PCR has been described^{12,13}. For transient transfection, the SLC-1 cDNA subcloned into pcDNA 3.1 (+) was transfected with G_q chimaeras into CHO dhfr (–) cells and HEK 293-T cells by using LipofectAMINE PLUS transfection reagent and following the manufacturer's instructions (GIBCO-BRL). For stable transfection, the SLC-1 cDNA subcloned into pcDNA 3.1 (+) was transfected into CHO dhfr (–) cells by the calcium phosphate method²⁸, and stable cell lines were established. To confirm that the plasmid SLC-1 had been integrated into the CHO genomic DNA, we analysed these cell lines by northern blot and chose one clone for further experiments.

Purification of SLC-1 endogenous ligand. 400 g frozen rat brain (Pel-Freez) was extracted in 1 M acetic acid and centrifuged at 20,000g for 15 min at 4 °C. The resulting supernatant was precipitated with acetone and extracted with diethylether. The aqueous phase was concentrated and loaded onto a C18 reverse-phase column (PrePAK-Delta-Pak, 25 × 100 mm; Waters) and eluted with a linear gradient of 5–48% CH₃CN in 0.1% trifluoroacetic acid (TFA). The active fractions were pooled and further purified on a cation-exchange column AP-1/SP-8HR (Waters) with a linear gradient of 0.15–0.35 M NaCl in 6 mM HCl and 30% CH₃CN. Active fractions were further purified on an analytical C18 Select B column (Merck) with a linear gradient of 21–33% CH₃CN in 0.1% TFA. Positive fractions were then serially fractionated in a SMART system on a Sephasil C8 SC2.1/10 column (Pharmacia) with a linear gradient of 36–42% CH₃CN in 0.1% TFA, a Sephasil C8 SC2.1/10 (Pharmacia) with a linear gradient of 33–48% CH₃CN in 0.1% heptafluorobutyric acid (HFBA), a μRPC C2/C18 SC2.1/10 column (Pharmacia) with a linear gradient of 34–35.1% CH₃CN in 0.1% TFA, and finally on a Sephasil C8 SC2.1/10 (Pharmacia) with a linear gradient of 26.4–27.6% CH₃CN in 0.1% TFA at a flow rate of 0.1 ml min^{–1}. Amino-acid sequences were determined in a pulse-linked automatic sequencer.

Intracellular calcium transient assay. We performed the assay as described²⁹. In brief, transfected or control cells were seeded into 96 wells at 5.5 × 10⁴ cells per well. The cells were loaded with Fluo-3 AM (Molecular Probes) in standard buffer solution (130 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 10 mM glucose, 0.45 mM KH₂PO₄, 0.4 mM Na₂HPO₄, 8 mM MgSO₄, 4.2 mM NaHCO₃, 20 mM HEPES and 10 μM probenecid) with 0.1% fetal bovine serum for 1 h at 37 °C, then washed with a standard buffer solution. Transient changes in [Ca²⁺]_i evoked by HPLC fractions were monitored using the FLIPR system

(Fluorometric Imaging Plate Reader; Molecular Devices) in 96-well plates at 488 nm for 210 s. For *in vitro* pharmacological characterization, we performed the same procedures using stably transfected CHO cells expressing rat SLC-1.

cAMP assay. SLC-1-expressing cells were plated in 24-well plates and grown to confluency. After removing the culture medium, we added variable amounts of synthetic MCH in a total volume of 0.3 ml of Dulbecco's modified Eagle's medium (containing 10 mM HEPES, 1 μM forskolin and 2 μM phosphodiesterase inhibitor Ro20-1724) and incubated the cells for 15 min at 37 °C. The medium was aspirated, the cells were extracted with 1 ml 70% ethanol and centrifuged to remove the debris, and the supernatant was lyophilized. We measured the cAMP content by competitive binding assay using antibodies to cAMP (Sigma) and [¹²⁵I]-cAMP (NEN).

Northern blot analysis. The 1.1-kb insert of SLC-1 was labelled with α³²P-dCTP and used as a probe in northern blot analysis. Northern blots containing 3 μg of poly(A)⁺ RNA from various rat tissues were hybridized and washed under high-stringency conditions. Blots were exposed to Kodak X-OMAT film at –80 °C with two intensifying screens.

In situ hybridization. A 0.6-kb BamHI–XbaI fragment of human SLC-1 cDNA was generated and subcloned into the pBluescript II SK (+) vector. The homology between human and rat sequences is 92% in this fragment. We generated sense and antisense riboprobes by T7 and T3 RNA polymerases, respectively, in the presence of ³⁵S-UTP. *In situ* hybridization to adult rat whole-brain sections was performed as described³⁰.

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Therapy of tuberculosis in mice by DNA vaccination

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Mycobacterium tuberculosis continues to kill about 3 million people every year¹, more than any other single infectious agent. This is attributed primarily to an inadequate immune response towards infecting bacteria, which suffer growth inhibition rather than death and subsequently multiply catastrophically. Although the bacillus Calmette–Guérin (BCG) vaccine is widely used, it has major limitations as a preventative measure². In addition, effective treatment requires that patients take large doses of antibacterial drug combinations for at least 6 months after diagnosis³, which is difficult to achieve in many parts of the world and is further restricted by the emergence of multidrug-resistant strains of *M. tuberculosis*. In these circumstances, immunotherapy to boost the efficiency of the immune system in infected patients could be a valuable adjunct to antibacterial chemotherapy⁴. Here we show in mice that DNA vaccines, initially designed to prevent infection, can also have a pronounced therapeutic action. In heavily infected mice, DNA vaccinations can switch the immune response from one that is relatively inefficient and gives bacterial stasis to one that kills bacteria. Application of such immunotherapy in conjunction with conventional chemotherapeutic antibacterial drugs might result in faster or more certain cure of the disease in humans.

DNA vaccination protects mice against subsequent challenge with tuberculosis^{5–7} by establishing a cellular immune response that is dominated by antigen-specific T lymphocytes that both produce interferon- γ (IFN- γ) and are cytotoxic towards infected cells (a type-1 cellular immune response). Both of these functions are probably required for maximally effective antimycobacterial immunity in mice⁸ and in humans^{9,10}. These responses are particularly favoured by DNA vaccines¹¹. In contrast, antigen-specific T cells that produce interleukin-4 (IL-4) and are not cytotoxic (a type-2 cellular immune response) are abundant during infection with *M. tuberculosis*^{12,13}. Such type-2 responses do not contribute to protection⁸ and so a shift in the balance towards type-1 responses might be beneficial. Furthermore, because effective immunity to tuberculosis probably depends on possessing appropriate responses to multiple antigens, a broad shift in the phenotype of T cells

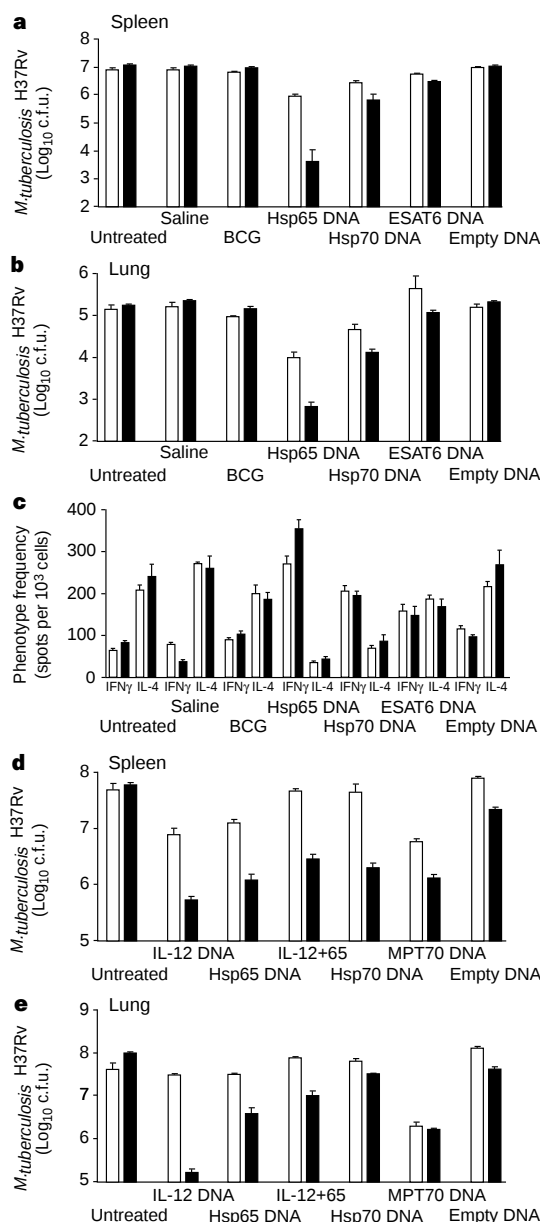


Figure 1 Therapeutic effects of DNA vaccination against an established *M. tuberculosis* H37Rv infection. **a–c**, Declining bacterial numbers and altered T-cell profiles after treatment with DNA encoding antigen Hsp65. Treatment commenced 8 weeks after intravenous injection of 5×10^5 live bacteria, when there were about 2×10^6 and 8×10^4 bacteria in spleens and lungs, respectively. Four intramuscular injections of plasmid DNA were given at 2-week intervals. The numbers of live bacteria in spleen (**a**) and in lung (**b**) 2 months (open bars) and 5 months (filled bars) after treatment commenced are shown as mean $\pm$ standard deviation (s.d.) ($n = 5$). Frequencies of lymph node cells (**c**) producing IFN- γ or IL-4 at 2 months (open bars) or at 5 months (solid bars) after treatment commenced were assayed by ELISPOT. Results are mean $\pm$ s.d. from triplicate determinations on cells pooled from groups of five mice. The effects of Hsp65 and Hsp70 DNAs were highly significant in both organs compared with empty plasmid or untreated controls at both time points (Student's *t*-tests, $P < 0.001$). **d, e**, Treatment with DNA expressing either antigen MPT70 or IL-12 also caused bacterial decline in spleen (**d**) and lung (**e**). Treatment commenced 8 weeks after intraperitoneal injection of 10^6 live bacteria when there were about 3×10^7 and 4×10^7 bacteria in spleen and lung, respectively. Counts of bacteria at 8 weeks (open bars) and 11 weeks (solid bars) after commencement of treatment are shown as mean $\pm$ s.d. ($n = 5$). The effects of IL-12, MPT70 and Hsp65 DNAs were highly significant in both organs at 11 weeks compared with either empty DNA or untreated controls (Student's *t*-tests, $P < 0.001$).

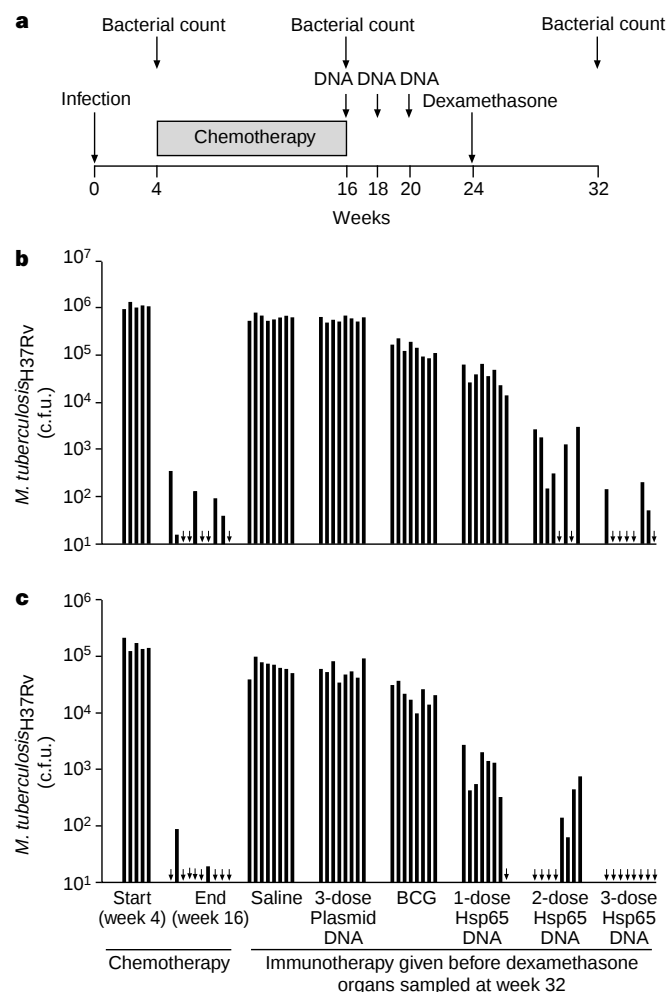


Figure 2 Elimination of residual *M. tuberculosis* by DNA vaccination after chemotherapy. Mice were given isoniazid plus pyrazinamide chemotherapy in the diet from week 4 of infection to week 16. Hsp65 DNA vaccine was given one, two or three times at 2-week intervals after completion of chemotherapy, followed by immunosuppression by dexamethasone treatment at 24 weeks. **a**, The schedule of treatments and sampling. **b**, **c**, Bacterial counts from the organs of individual mice; arrows indicate organs with counts below the level of detection. At week 16, only a few bacteria were detected in the spleens (**a**) of 5/10 mice and lungs (**b**) of 2/10 mice; no bacteria were detected in the remainder. Bacterial counts at 32 weeks are shown for groups of eight mice that received one, two or three doses of Hsp65 DNA, three doses of empty plasmid or saline, or a single injection of BCG at week 16. At week 32, only a few bacteria were detected in the spleens of 3/8 mice and lungs of 0/8 mice that had received three doses of the DNA vaccine before dexamethasone.

responding to many different antigens might be needed for maximum therapeutic effect.

DNA vaccination may be particularly suited to achieve such a shift because of an immunostimulatory 'adjuvant' effect of plasmid DNA itself¹⁴. Indeed, prophylactic DNA vaccination against tuberculosis increased the proportion of spleen T cells that expressed an activated (CD44hi) phenotype (associated with the type-1 response) from about 12 to 40%, even though less than one in a thousand were specific for the immunizing antigen (a heat-shock protein with a relative molecular mass of 65,000 (M_r 65K): antigen Hsp65)⁸. Therapeutic effects of DNA vaccination against *Mycoplasma pulmonis* have also been reported^{15,16}. Therefore we tested whether DNA vaccination could have a beneficial effect and enhance the type-1 response during the course of tuberculosis.

An infection initiated by intravenous injection of virulent *M. tuberculosis* H37Rv was allowed to develop for 8 weeks, during which time the numbers of bacteria in the internal organs increased

slowly by about four-fold. Mice were then given a series of four doses of plasmid DNA intramuscularly at 2-week intervals. The numbers of live bacteria in spleen and lungs declined rapidly 2 months and 5 months after the first dose of DNA when the plasmid expressed antigen Hsp65 (Fig. 1a, b). Much smaller effects were obtained with plasmid expressing other mycobacterial antigens (the 70K heat-shock protein or the 6K early secretory antigen: Hsp70, ESAT6) and this parallels the lower efficacy of these antigens in prophylactic DNA vaccination¹⁷. There was virtually no change induced with plasmid that contained no insert, or by a single dose of the standard BCG vaccine, compared with untreated or saline-treated control mice. To test if the therapeutic vaccination had modified T-cell priming we estimated the frequencies of T cells that typified type-1 or type-2 immune responses in the lymph nodes of treated and untreated mice. The therapeutic effect of DNA vaccination was indeed associated with a switch from a predominantly type-2 to a predominantly type-1 response (Fig. 1c). DNA-vaccine therapy was equally effective against an established infection with a clinical isolate of *M. tuberculosis* (CLS52) that is resistant to isoniazid, one of the main chemotherapeutic drugs, and an identical change in T-cell phenotypes was observed (data not shown).

This unexpected efficacy of DNA-vaccine therapy was confirmed in an experiment in which a higher dose of bacteria was given and the bacteria attained much higher levels of infection (Fig. 1d, e). Although the magnitude of the observed therapeutic effect of plasmid expressing Hsp65 was somewhat less, the experiment had to be curtailed because the control mice that were untreated or received empty plasmid began to show clinical signs of advanced tuberculosis. In this experiment, either a plasmid expressing a 22K mycobacterial secretory antigen (MPT70) that may be upregulated in infection^{18,19}, or a plasmid expressing interleukin-12 (IL-12; see below), was at least as beneficial as a plasmid expressing Hsp65.

Much of the adjuvant effect of DNA vaccines is due to their content of non-methylated CpG sequences that induce IL-12 secretion by cells engaged in antigen presentation¹⁴. IL-12 selectively promotes the acquisition of a type-1 phenotype and of cytotoxicity during priming and maturation of adjacent lymphocytes, irrespective of their antigen specificity²⁰. Furthermore, this cytokine is crucial in the development of protective immunity against tuberculosis^{10,21–23}. Therefore we also tested the therapeutic effect of a plasmid that expressed IL-12 instead of a mycobacterial antigen. This plasmid caused the greatest reductions in bacterial numbers by 11 weeks (Student's *t*-tests, $P < 0.001$), although co-delivery of plasmids expressing IL-12 and Hsp65 as a 50:50 mixture resulted in antagonism and reduced therapeutic efficacy. The basis of such interactions between plasmids and the identities of the therapeutic T cells elicited by plasmids expressing either antigens or IL-12 are the subject of further investigation.

When modern chemotherapy fails, it most often does so as a result of re-growth of fully drug-sensitive bacteria that have persisted in a non-replicating and physiologically drug-resistant form^{3,24}. We tested whether the addition of DNA vaccination at the end of chemotherapy could reduce this problem. Three intramuscular doses of Hsp65 DNA vaccine appeared to eliminate residual bacteria from a proportion of the mice when the DNA was given after chemotherapy (Fig. 2); injections of immunosuppressive corticosteroid were unable to reactivate bacterial growth, indicating a sterilizing effect²⁵. The proportion of mice with spleens or lungs that appeared to be sterile increased significantly as the number of DNA doses was increased (trend χ^2 test, $P < 0.001$).

In the models of tuberculosis tested here, infection was established by a large systemic dose of bacteria. This not only presented a severe challenge for therapy, but it also reflected the usual aetiology of human tuberculosis. Blood-borne dissemination of bacteria from the primary pulmonary focus of infection is needed to establish the secondary foci that are the main cause of disease²⁶. However, mice have a relatively high innate resistance to tuberculosis and natural

transmission of most human tuberculosis is airborne, by inhalation of small numbers of bacteria. Consequently, it will be appropriate to evaluate immunotherapy in other more susceptible animal models and after airborne infection. In addition, the effect of giving chemotherapy and immunotherapy simultaneously should be assessed, as this would be most useful clinically. Nevertheless, the present findings strengthen the case for evaluating immunotherapy as an adjunct to chemotherapy⁴. Furthermore, persistence of the bacteria can occur after natural resolution of the initial infection in humans. Bacterial re-growth many years later when the immune system declines accounts for most of the tuberculosis found in developed countries²⁷. We can speculate therefore that similar vaccines used prophylactically and therapeutically might be able to both prevent establishment of this persistent state and eliminate it if it is already established.

Note added in proof: Results of preliminary experiments in which Hsp65 DNA therapy was given 8 weeks after aerosol infection of either mice or guinea pigs have indicated substantial therapeutic benefits in these models also, decreasing c.f.u. and prolonging survival respectively. □

Methods

Therapy of established infection by DNA vaccination. Female Balb/c mice aged 6–8 weeks were infected by either intravenous or intraperitoneal injection with *M. tuberculosis* H37Rv. DNA vaccination was initiated after 8 weeks and was done by injection of 50 µg of plasmid DNA in 50 µl saline into the quadriceps muscle of each hind leg, on four occasions at 2-week intervals. When plasmid mixtures were given, they were injected using the same total amount of DNA per dose (50 µg). Control mice received empty plasmid or saline injections only or a single subcutaneous injection of 10⁵ live BCG in 50 µl saline at 8 weeks. Mice were killed at intervals and bacteria in internal organs were counted as colony forming units (c.f.u.) on 7H11 medium⁸.

Plasmids. DNA sequences encoding *M. leprae* Hsp65, *M. tuberculosis* Hsp70 or *M. tuberculosis* ESAT6 antigens were cloned into pCDNA3 (Invitrogen)¹⁷. For the experiment shown in Fig. 1d, e, we used pCMV4. This differs from pCDNA3 by containing, in addition to the promoter of the cytomegalovirus (CMV) immediate early gene, the first intron (IE-1), cloned from hCMV strain 169 as a HindIII-terminated 0.9-kilobase product by a polymerase chain reaction; ref. 28). Inclusion of the intron can give higher levels of antigen expression²⁹. Plasmid expressing murine IL-12 (pIRES muIL-12) was constructed on a backbone that was similar to pCMV4 and used an internal ribosome entry site to express both the p35 and p40 subunits from the CMV IE-1 promoter/enhancer³⁰.

T-cell frequencies. Lymphocytes were obtained from the pooled inguinal and mesenteric lymph nodes. Plastic- and nylon-adherent cells were removed and the frequencies of cells producing IFN-γ (type-1 phenotype) or producing IL-4 (type-2 phenotype) in the presence of phorbolmyristic acid (10 ng ml⁻¹) and monoclonal antibody against CD3 (YCD3-1, 50 ng ml⁻¹; Gibco-BRL) were determined by limiting dilution ELISPOT analysis⁸.

Elimination of persistent bacteria by DNA vaccination. Four weeks after the initiation of infection in Balb/c mice by intravenous injection of 5.4×10^6 live *M. tuberculosis* H37Rv, mice were placed on a standard diet supplemented with isoniazid (25 mg kg⁻¹) and pyrazinamide (1,000 mg kg⁻¹) for 12 weeks and the decline in c.f.u. in spleens and lungs was monitored. Mice were then given one, two or three intramuscular doses of 100 µg Hsp65 DNA vaccine at 2-week intervals, three doses of empty plasmid, or a single subcutaneous dose of live BCG. Eight weeks after the first dose of DNA, three subcutaneous injections of dexamethasone (6 mg kg⁻¹) were given 2 days apart and bacterial c.f.u. in lungs and spleens were measured after a further 8 weeks.

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ovo/svb integrates Wingless and DER pathways to control epidermis differentiation

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In *Drosophila*, as in mammals, epidermal differentiation is controlled by signalling cascades that include Wnt proteins^{2,3} and the *ovo/shavenbaby* (*svb*) family of zinc-finger transcription factors^{4–6}. *Ovo/svb* is a complex gene with two genetic functions corresponding to separate control regions: *ovo* is required for female germ-line development and *svb* for epidermal morphogenesis^{7,8}. In the *Drosophila* embryo, the ventral epidermis consists of the segmental alternance of two major cell types that produce either naked cuticle or cytoplasmic extrusions known as denticles. Wingless signalling specifies smooth cells that produce naked cuticle⁹, whereas the activation of the *Drosophila* epidermal growth factor (EGF) receptor (DER) leads to the production of denticles¹⁰. Here we show that expression of the *ovo/svb* gene controls the choice between these cell fates. We find that *svb* is a key selector gene that, cell autonomously, directs cytoskeletal

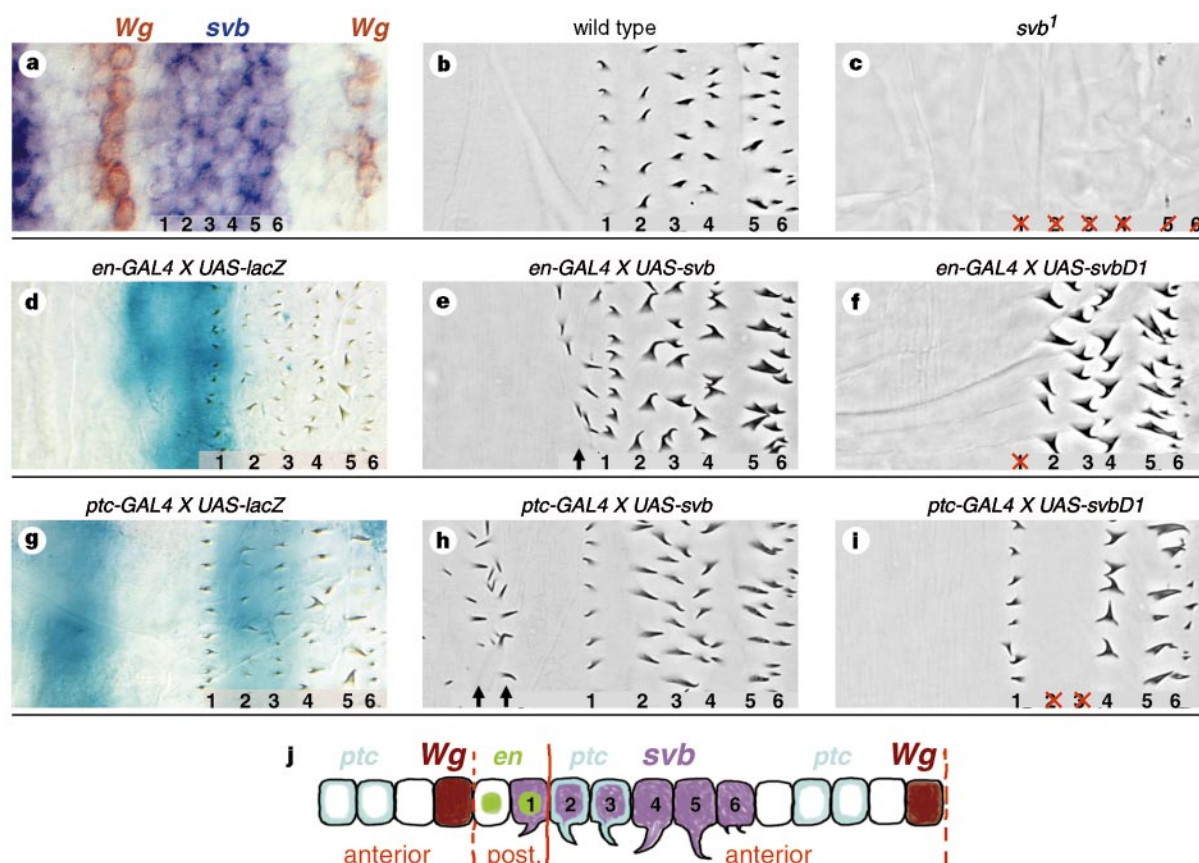


Figure 1 *svb* activity is necessary and sufficient to promote denticle formation. **a**, Ventral epidermis of wild-type stage 11 embryo stained for Wg protein (brown) and *svb* transcripts (purple). Anterior is left in all pictures. *svb* is expressed in the six rows of epidermal cells forming denticles. **b**, Wild-type cuticle. **c**, *svb*¹ cuticle. Embryos lacking zygotic *svb* function present almost exclusively naked cuticle, with only sparse atrophied denticles in the posterior-most rows (5, 6) of each belt. **d–f**, Engrailed-Gal4 driver was used to direct the expression of UAS constructs in the posterior compartment of each segment. **d**, Embryos carrying UAS-lacZ, stained for β-galactosidase activity (blue). **e**, Expression of UAS-*svb* with en-Gal4 induces the formation of an extra row of denticles in anterior En cells (arrow). The morphology of denticles normally present in the posterior En cell row is unaffected by *svb* overexpression. **f**, Expression of the dominant negative protein

Ovo/svbD1 antagonizes *svb*. In embryos expressing UAS-*svbD1* with en-Gal4, both cell rows of the posterior compartment make naked cuticle. **g–i**, Embryos carrying ptc-Gal4 driver and UAS-lacZ (**g**), UAS-*svb* (**h**), or UAS-*svbD1* (**i**). **g**, β-galactosidase staining. Ectopic expression of UAS-*svb* in naked cuticle produces ectopic denticles (arrows) (**h**), whereas expression of UAS-*svbD1* prevents denticle formation in the corresponding rows of cells (rows 2, 3) (**i**). Numbering refers to denticle rows, arrows to ectopic denticle rows, and red bars to extra naked cuticle. **j**, Schematic representation of the ventral epidermis with the expression patterns of *en* (green), *ptc* (blue), *wg* (brown) and *svb* (purple) relative to the position of denticle formation in abdominal segments. Segmental and parasegmental borders are represented by plain and dotted red lines, respectively.

modifications producing the denticle. The DER pathway promotes denticle formation by activating *svb* expression. Conversely, Wingless promotes the smooth cell fate through the transcriptional repression of *svb* by the bipartite nuclear factor Armadillo/dTcf. Our data indicate that transcriptional regulation of *svb* integrates inputs from the Wingless and DER pathways and controls epidermal differentiation.

Human and mouse orthologues of *ovo/svb* have been identified⁴. Targeted disruption of mouse *movo-1* inhibits gonadal development and also impairs hair formation⁴, indicating conservation of *svb* function during evolution. This implies more parallels between vertebrate and invertebrate epidermal differentiation than previously suspected. Supporting this hypothesis, β-catenin, a member of the Wnt signalling pathway, directs epidermal patterning in vertebrates² as it does in *Drosophila*³. However, downstream targets of signalling cascades in this process have remained elusive. We now show that both the Wingless and DER pathways determine the pattern of epidermal differentiation through the transcriptional regulation of *svb* expression. We find that *svb* is a selector gene responsible for denticle production, and that the on/off status of *svb* transcription accounts for denticle and naked cuticle formation, respectively.

In the *Drosophila* embryo, after the last wave of mitosis, *svb* is

expressed in a striped pattern in the ventral epidermis^{7,8}. Localization of *svb* transcripts relative to molecular segmentation landmarks (Fig. 1a and data not shown) shows that *svb* is expressed in the six rows of denticle-producing cells. Reducing *svb* expression leads to the transformation of denticle to naked cuticle (Fig. 1b, c), without affecting either segmentation or cell division (data not shown). Therefore, *svb* is expressed in each cell that will make a denticle and its function is required for denticle formation. The posterior compartment of each segment is composed of two rows of cells expressing the selector homeoprotein Engrailed (En), and only the posterior row of En cells expresses *svb* and gives rise to denticles^{11–13} (Fig. 1j). To test whether *svb* expression is sufficient to force anterior En cells to produce denticles instead of smooth cuticle, we drove expression of *svb* in En cells using the conditional UAS/Gal4 system¹⁴ (Fig. 1d). Whereas *svb* overexpression driven by en-Gal4 has no effect on denticle morphology in the posterior row of En cells, it induces an extra row of denticles in anterior En cells (Fig. 1e). This indicates that the on/off status of *svb* expression is directly related to the difference in cell fate between anterior and posterior En cells. If this hypothesis is correct, eliminating *svb* function in the posterior En cells should transform them to smooth cells. The *ovoD1* mutation, which exerts a dominant-negative (antimorphic)

effect on *ovo/svb* function in the germ line, has been molecularly characterized¹⁵. We generated transgenic lines allowing the expression of this dominant-negative product in the epidermis (UAS-*svbD1*). *en*-Gal4 embryos carrying UAS-*svbD1* lack the first row of each abdominal denticle belt (Fig. 1f). Antagonizing *svb* function in the posterior *En* cells is therefore sufficient to switch cells from the denticle to smooth fate. Our data indicate that *svb* is necessary and sufficient to trigger the cytoskeletal rearrangement¹⁶ responsible for denticle formation in the epidermis.

To analyse other epidermal cell types, we tested the action of *svb* in the anterior compartment. Using *ptc*-Gal4, *svb* expression can be driven in two separate stripes of epidermal cells in each segment: one stripe consists of two rows of cells that give rise to naked cuticle, and the other stripe corresponds to denticle rows 2 and 3 (Fig. 1g, j). *ptc*-Gal4-driven expression of *svb* causes the production of ectopic denticles instead of naked cuticle, but it does not affect the normal denticle rows (Fig. 1h). Conversely, expression of UAS-*svbD1* has no effect on naked cuticle, but prevents denticle formation in a cell-autonomous fashion (Fig. 1i). Similar results were obtained in all cells of the embryonic ventral epidermis tested, using a variety of Gal4 drivers. The denticle production induced by ectopic *svb* expression does not require endogenous *svb* activity and denticle morphology appears to be determined independently by positional information (data not shown and see below). These data establish that *svb* acts as a general switch for epidermis differentiation, in which *svb* function is required and sufficient to instruct the cell to make a denticle.

In addition to its role during segmentation, Wingless (Wg) is the determinant of naked cuticle: *wg* inactivation leads to the formation of a continuous lawn of denticles, whereas ectopic Wg expression results in naked embryos^{3,9}. Thus, *wg* and *svb* have opposite effects on epidermal cell shape, indicating that either *svb* represses *wg* action to trigger the denticle fate, or *wg* represses *svb* to permit naked cuticle differentiation. To distinguish between these two possibilities, we generated embryos lacking both *wg* and *svb* functions. These *wg/svb* double-mutant embryos display an epidermal phenotype characteristic of *svb* (Fig. 2a, b), showing that the formation of extra naked cuticle caused by *svb* mutation does not require Wg activity. This indicates that the effect of Wg on naked cuticle determination is mediated by repression of *svb*, a conclusion further supported by the ectopic activation of *svb* transcription that is observed in *wg* mutant embryos (Fig. 2c). Signal transduction triggered by Wg stabilizes cytosolic Armadillo¹⁷ (Arm). As Arm interacts with E-Cadherin¹⁸, a component of adherens junctions, a direct effect of the Wg pathway on the cytoskeleton cannot be formally excluded¹⁹. Amino-terminal truncation of the Arm protein (ArmS10)²⁰, in a region that is highly phosphorylated and frequently mutated in human colorectal cancers¹⁸, leads to stabilization of Arm, thus mimicking constitutive Wg signalling. Ubiquitous ArmS10 expression induces naked cuticle²⁰. We show here that it does so by repressing *svb* expression (Fig. 2d, f). Transcriptional repression of *svb* can fully account for the ArmS10 naked phenotype, as Gal4-mediated ubiquitous expression of *svb* in embryos overexpressing ArmS10 can restore wild-type denticles and produce ectopic denticles (Fig. 2e). Arm associates with dTcf/pangolin²¹ to form a bipartite nuclear factor that regulates, either positively or negatively, the transcription of Wg target genes^{22–24}. A dTcf molecule lacking the putative Arm-binding domain (dTcfDN) behaves as a dominant negative effector of the Wg pathway²³, and embryos expressing dTcfDN have a ventral epidermis covered with denticles. As is the case for Wg mutations, homogeneous dTcfDN expression also induces ectopic *svb* expression throughout the epidermis (Fig. 2i). Denticle formation induced by dTcfDN can be suppressed either by mutations in *svb* (Fig. 2h) or by antagonizing *svb* function using UAS-*svbD1*. Thus, the action of Wg signalling on smooth-cell fate specification is mediated by the nuclear transcriptional repression of *svb*, and this confirms that naked cuticle formation is not the result of a direct effect of the Wg pathway on the cell

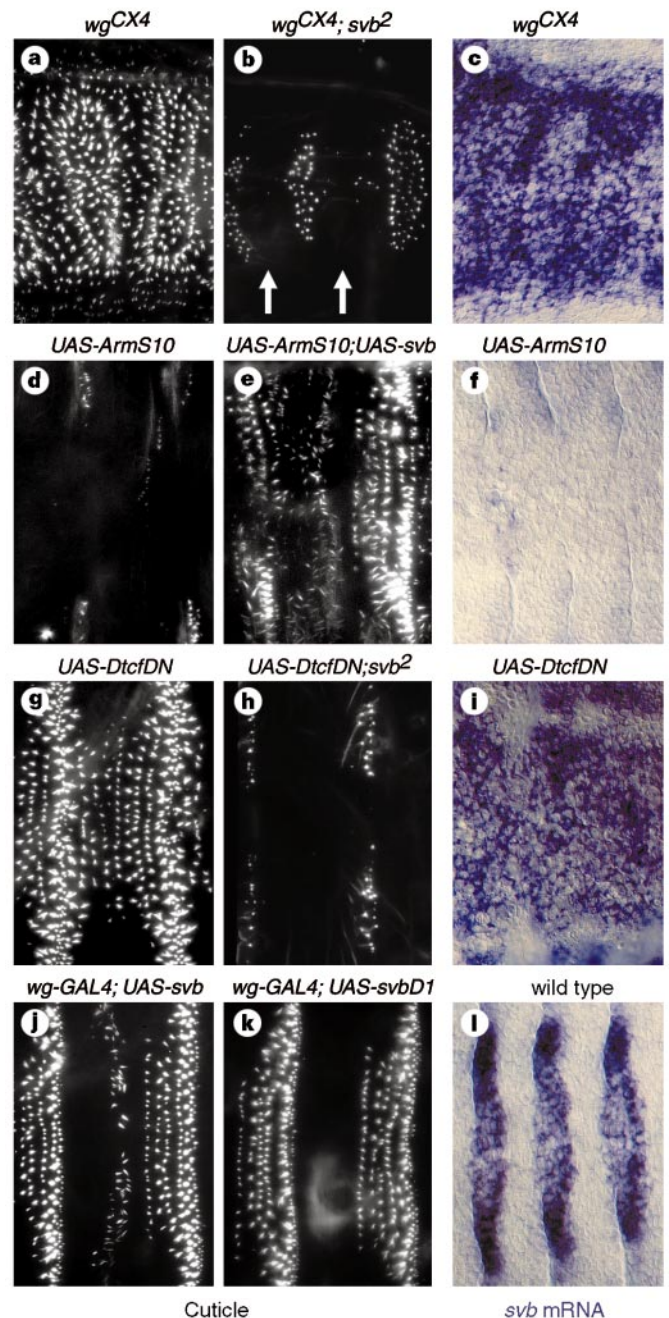


Figure 2 The Wg pathway represses *svb* transcription. Ventral views, anterior to the left. **a**, *wg* null mutation (*wg*^{CX4}) transforms the whole ventral epidermis towards the denticle fate. **b**, *svb* mutation also prevents denticle formation in a *wg*-null context: *wg*^{CX4}/*svb*² double mutant embryos produce naked cuticle (arrows); some residual abortive denticles are characteristic of the *svb*² allele. **c**, *wg* mutation leads to expression of *svb* messenger RNA in the entire ventral epidermis. **d–i**, the E22C-Gal4 driver was used to ubiquitously express UAS constructs. **d**, UAS-ArmS10 ubiquitous expression, mimicking constitutive action of the Wg pathway, produces a naked phenotype. **e**, Uniform expression of UAS-*svb* restores denticle formation in UAS-ArmS10 embryos. **f**, Ubiquitous expression of ArmS10 eliminates *svb* transcription in the ventral epidermis. **g**, dTcfDN acts as a nuclear repressor of the Wg pathway, leading to the formation of extra denticles. **h**, Both normal and dTcfDN-induced denticles are suppressed by *svb* mutation. **i**, UAS-dTcfDN expression, blocking the Wg pathway, results in ectopic expression of *svb*. **j**, **k**, embryos carrying a *wg*-Gal4 driver. *wg*-Gal4-driven expression of UAS-*svb* produces denticles in the *wg*-expressing cells (**j**), whereas expression of UAS-*svbD1* in the same cells results in embryos with a wild-type cuticle (**k**). **l**, *svb* expression in a wild-type embryo.

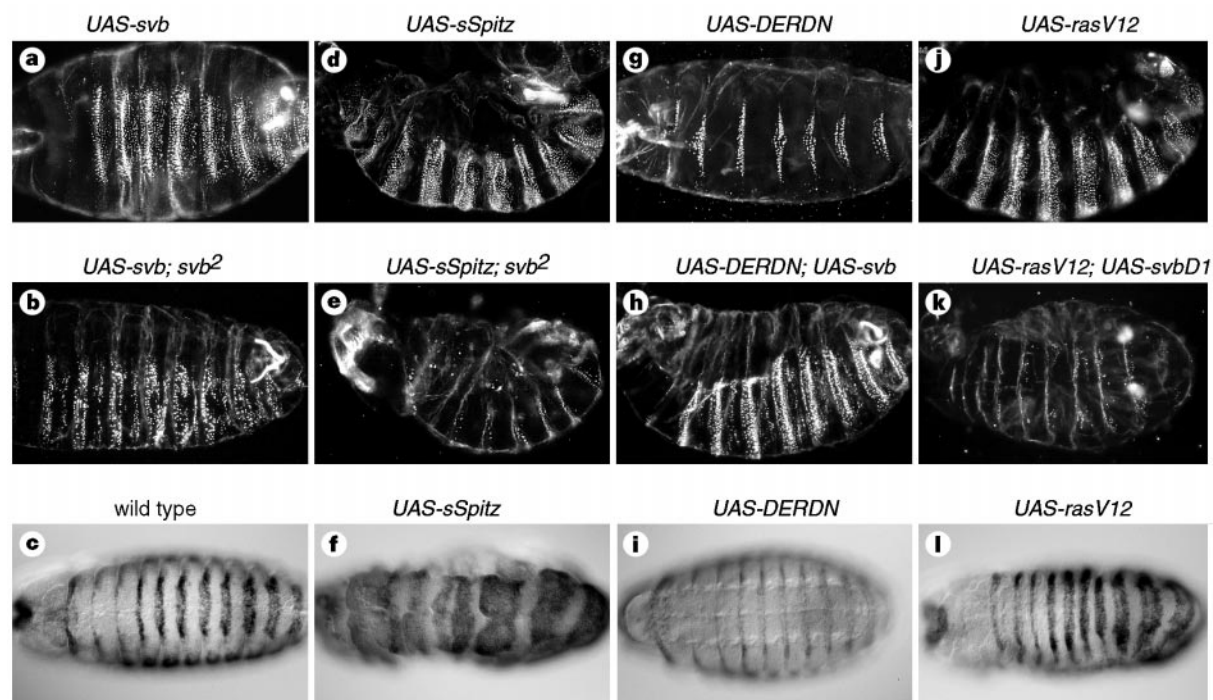


Figure 3 The DER signalling pathway directs denticle formation by activating *svb* expression. *ptc*-Gal4-mediated expression of UAS-*svb* produces ectopic denticles in both wild-type (a) and *svb*² embryos (b). Ventral views, anterior is left in all pictures. c, *svb* expression in wild-type embryos. d–l, E22C or 69B Gal4 drivers were used to direct ubiquitous expression of UAS constructs. Ectopic denticles produced by the ubiquitous expression of UAS-sSpitz (d) are suppressed by *svb* mutation (e) and are due to ectopic *svb* expression (f). Formation of extra naked

cuticle induced by UAS-DERDN (g) is counteracted by co-expression of UAS-*svb* (h) and is mediated by the restriction of *svb* transcription to the posterior rows 5 and 6 (i). Formation of extra denticles upon ectopic activation of the DER pathway by UAS-rasV12 in a wild-type background (j) is suppressed by co-expression of UAS-*svb*D1 (k). The *svb* expression domain is enlarged in UAS-rasV12-expressing embryos (l). a, b, d, e, g, h, j, k: cuticle preparation. c, f, i, l: *svb* mRNA *in situ* hybridization.

cytoskeleton²⁵. Finally, that the Wg pathway represses *svb* expression, rather than downstream cellular events positively regulated by *svb*, is further supported by the production of ectopic denticles in the cells subjected to the highest levels of Wg signalling, the Wg-expressing cells themselves, when *svb* is expressed under the control of the *wg* promoter (Fig. 2j). These results establish that *svb* is transcriptionally repressed by the Wg pathway, and that this repression is a key function of Wg in the control of epidermal cell morphogenesis.

Although repression of *svb* accounts for the role of Wg in naked cuticle determination, what are the mechanisms leading to *svb* activation in the cells forming denticles? The simplest hypothesis is that *svb* expression, and therefore denticle formation, corresponds to an epidermal-cell ground state, with negative control by Wg being sufficient to define the alternate pattern of naked cuticle and denticles. Alternatively, *svb* expression may be specifically activated in denticle cells. We investigated whether the EGF/DER pathway, which is required for the formation of the four anterior denticle rows¹⁰, is involved in transcriptional activation of *svb* in these cells.

In the epidermis, homogeneous expression of an activated (secreted) form of Spitz (sSpitz), a putative EGF-like ligand of the DER receptor²⁶, induces the formation of ectopic rows of denticles⁶. These ectopic denticles, like the wild-type (but unlike those induced by UAS-*svb*) are suppressed in *svb* mutant embryos (Fig. 3a–e). In addition, UAS-sSpi embryos display a strong ectopic expression of *svb*, prefiguring the pattern of the resulting extra denticles (Fig. 3f). Antagonizing DER function by overexpressing the dominant negative DERDN protein restricts denticle formation to the two posterior rows of each segment (rows 5 and 6) in otherwise wild-type embryos¹⁰. However, it does not prevent the denticle formation induced by UAS-*svb* (Fig. 3g, h). Furthermore, antagonizing DER receptor function leads to the restriction of *svb* expression to the two

cell rows (Fig. 3i) corresponding to the pattern of the remaining denticles¹⁰. Finally, activation of the DER pathway using intracellular components, such as RAS-V12, an activated form of RAS^{27,28}, gives results similar to those obtained using the putative ligand, but by a cell-autonomous mechanism. Again, the production of ectopic denticles requires *svb* function and is mediated by the ectopic activation of *svb* transcription (Fig. 3j–l). These data show that the DER pathway positively regulates *svb* transcription and that this regulation is crucial for the pattern of denticle formation.

Whereas the pattern of DER pathway activation correlates with *svb* expression in cells of denticle rows 1–4, and Wg is expressed in cells making naked cuticle (Fig. 4a–c), both Wnt and EGF-like factors are secreted molecules that can act at a distance²⁹, raising the question of how a given epidermal cell integrates these antagonistic signals to turn *svb* transcription on or off. Unlike inactivation of the Wg pathway, ectopic activation of the DER pathway throughout the epidermis does not produce a continuous lawn of denticles¹⁰. In these embryos, we show that *svb* transcription is extended to cells forming ectopic denticles (Fig. 4d–f). However, although the DER pathway is activated everywhere, as revealed by phosphorylation of the ERK1 MAP-kinase³⁰, the transcription of *svb* is not turned on in cells displaying high Wg activity (Fig. 4d, e). That repression by Wg can overcome DER-mediated activation of *svb* transcription is confirmed by the further expanded expression of *svb* in embryos with reduced Wg activity, in addition to UAS-sSpi expression (results not shown). Together, these results show that cell-fate control during epidermal differentiation is achieved by the precise regulation of the expression of the *Svb* transcription factor by two antagonistic signalling pathways. Activation of the DER pathway results in the transcriptional activation of *svb*, but this is counteracted by high levels of Wg signalling. Therefore, in an epidermal cell, the regulatory region of *svb* integrates these opposing signals, leading in a simple binary fashion to an on or off state of trans-

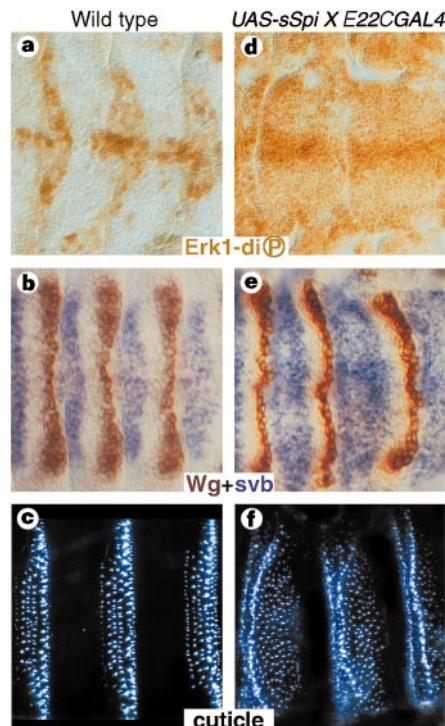


Figure 4 *svb* transcription integrates the antagonist signals from the Wg and DER pathways. **a**, In a wild-type embryo, the phosphorylation pattern of ERK1 MAP-kinase, visualized by anti-diphospho-ERK1 antibody staining, reveals activation of the DER pathway in four cell rows correlating with *svb* transcription. Ventral view, anterior to the left. **b**, The domains of expression of the Wg protein (brown) and *svb* transcripts (purple) are mutually exclusive. **c**, Wild-type cuticle. **d-f**, Uniform expression of UAS-*sSpi* (driven by E22C-Gal4) leads to ectopic activation of the DER pathway throughout the ventral epidermis (**d**). The resulting ectopic *svb* expression is limited by high Wg activity (**e**) and prefigures the position of ectopic denticles (**f**). **a, d**, Anti-diphospho-ERK1 staining; **b, e**, Double staining for Wg protein (brown) and *svb* mRNA (purple); **c, f**, Cuticle preparation.

cription, depending on the relative signalling activities received. Repression of *svb* results in naked cuticle formation, whereas expression of *svb* triggers the cellular mechanisms responsible for denticle differentiation.

Our data show that the tight regulation of *ovo/svb* expression by the Wnt and DER pathway is determinant for epidermal differentiation. The skin defects resulting from deregulation of Wnt signalling² or *m-ovo1* knock-out⁴ indicate that this regulatory network may be conserved in mammals. Therefore, we propose that, in both vertebrates and invertebrates, the control of *ovo/svb* expression represents a major aspect of signalling pathway function in epidermal differentiation. □

Methods

Gal4 driver lines. E22C-Gal4 and 69B-Gal4 driver lines were used for homogeneous expression throughout the epidermis, en-Gal4 and ptc-Gal4 were used for expression in the corresponding rows of epidermal cells.

Gal4-responsive lines. UAS-*svb* was made by subcloning an embryonic *ovo/svb* complementary DNA as an *HpaI/NotI* fragment in pUAST. UAS-*svbD1* was made by introducing the *ovoD1* point mutation, leading to the expression of an N-terminal extended form that acts as a transcriptional repressor. UAS-ArmS10, UAS-DtcfDN, UAS-Wg, UAS-RasV12, UAS-sSpi and UAS-DERDN were from M. Peifer, J. P. Vincent, D. Montell, M. Freeman and B. Shilo, respectively. Embryos co-expressing one of the above UAS constructs together with UAS-*svb* (or UAS-*svbD1*) were obtained from stocks made homozygous for both transgenes, using balancer chromosomes and UAS-*svb(D1)* insertions located on chromosomes II or III. For analysis of *svb/wg* double mutants, we generated the stock *svb²/FM7; wg^{CX4}/CyO*. We use the *svb²* hypomorphic allele,

which produces characteristic atrophic denticles in the posterior rows, to easily score embryos presenting the new phenotype, that is, 'football' shaped embryos (diagnostic of the *wg^{CX4}* null mutation) that display naked cuticle and atrophic denticles (characteristic of the *svb²* mutation). These embryos were observed at the expected frequency (1/7). Expression of UAS constructs in an *svb* mutant background was achieved using the following stocks: *svb,btd/FM7; E22C-GAL4/CyO* or *svb,btd/FM7; ptc-GAL4/ptcGAL4*. Thus, in addition to the identification of new phenotypes, the head defect phenotype due to the *btd* mutation allowed us to score for *svb* mutant embryos.

Staining and mounting of samples. Cuticles were mounted in Hoyer's/lactic acid and observed using phase-contrast and dark-field microscopy. Anti-Wg (monoclonal antibody from S. Cohen) and anti-diphospho-Erk1 (Sigma) antibody staining, as well as *svb in situ* hybridization, were performed according to standard procedures. Dissected epidermis was mounted in Aquapolymount (Polyscience).

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The cell-surface proteoglycan Dally regulates Wingless signalling in *Drosophila*

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Wingless (Wg) is a member of the Wnt family of growth factors, secreted proteins that control proliferation and differentiation during development. Studies in *Drosophila* have shown that responses to Wg require cell-surface heparan sulphate, a glycosaminoglycan component of proteoglycans^{1–4}. These findings suggest that a cell-surface proteoglycan is a component of a Wg/Wnt receptor complex. We demonstrate here that the protein encoded by the *division abnormally delayed* (*dally*) gene is a cell-surface, heparan-sulphate-modified proteoglycan^{5,6}. *dally* partial loss-of-function mutations compromise Wg-directed events, and disruption

of *dally* function with RNA interference produces phenotypes comparable to those found with RNA interference of *wg* or *frizzled* (*fz*)/*Dfz2* (ref. 7). Ectopic expression of Dally potentiates Wg signalling without altering levels of Wg and can rescue a *wg* partial loss-of-function mutant. We also show that *dally*, a regulator of Decapentaplegic (Dpp) signalling during post-embryonic development, has tissue-specific effects on Wg and Dpp signalling. Dally can therefore differentially influence signalling mediated by two growth factors, and may form a regulatory component of both Wg and Dpp receptor complexes.

dally was identified in a genetic screen for mutations affecting cell division patterning during visual system development in *Drosophila*; mutations in *dally* are pleiotropic, affecting antennal, eye, genitalia and wing morphogenesis⁵. The *dally* cDNA shows homology in the protein coding sequence to glypicans, a family of integral membrane proteoglycans discovered in vertebrates. We have performed experiments to determine directly whether Dally is a cell-surface heparan sulphate (HS)-modified proteoglycan and therefore a candidate for serving as a component of the Wg receptor. A haemagglutinin (HA) epitope-tagged form of Dally was expressed in *Drosophila* S2 cells to provide material. We examined whether Dally is found on the cell surface and whether, like the vertebrate glypicans, it bears a glycosylphosphatidylinositol (GPI) linkage. GPI-linked proteins are cleaved selectively by phosphatidylinositol-specific phospholipase C (PI-PLC); treatment of S2 cells expressing HA-Dally with PI-PLC quantitatively releases Dally from the cell surface, demonstrating its GPI linkage to the outer leaflet of the plasma membrane (Fig. 1a). To determine whether Dally bears glycosaminoglycan chains of the HS type, total proteins from HA-

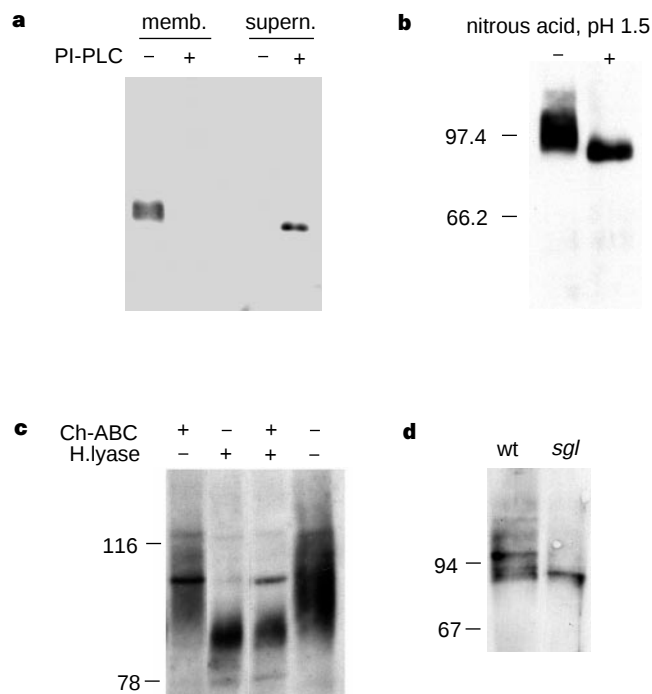


Figure 1 Biochemical characterization of HA-Dally. **a**, An HA-epitope tagged Dally expressed in *Drosophila* S2 cells is released from the cell surface by PI-PLC, indicating its GPI linkage. **b**, HA-Dally bears N-sulphated glucosamine residues. Low-pH treatment with nitrous acid removes HS chains for HA-Dally. **c**, HA-Dally bears sugar chains sensitive to heparin lyase II but not to chondroitinase ABC (Ch-ABC). The band seen in Ch-ABC-treated lanes represents non-specific binding of antibody to Ch-ABC. **d**, Glycosaminoglycan modification of wild-type (wt) Dally expressed in third-instar larvae requires *sgl* activity. Positions of marker proteins ($10^{-3}M_r$) are shown at the left.

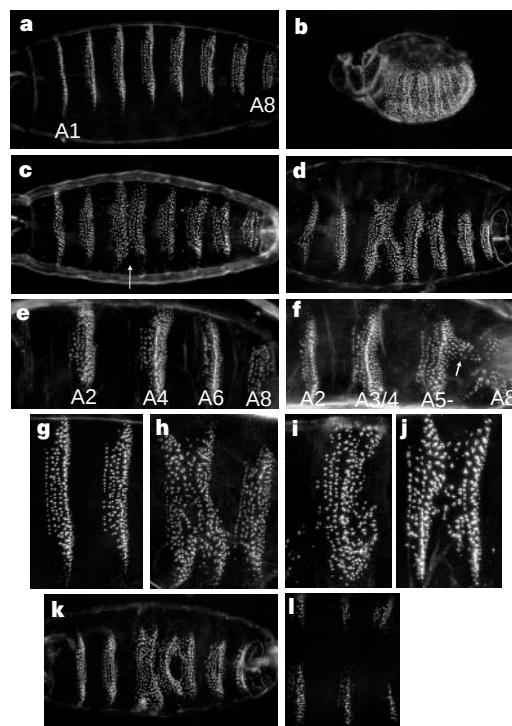


Figure 2 *dally* affects cuticle patterning in the embryo. **a**, A wild-type embryo (segments A1–A8 marked, all cuticles with anterior to the left). **b**, **c**, *wg*^{IL114} homozygotes (a temperature-sensitive allele) grown at the non-permissive temperature, 25°C (**b**), and at an intermediate temperature, 20°C (**c**), where some *wg* activity remains. **d**, *dally*^{ΔP-527}/*dally*^{P2}. **e**, *ftz*⁺ RNAi. **f**, *dally*⁺ RNAi. The arrow marks ectopic denticles; A3 and A4 are fused. **g**, Wild-type cuticle, high magnification. **h**, *dally*^{ΔP-527}/*dally*^{P2}, high magnification. **i**, *dally*⁺ RNAi, high magnification, showing fused denticle bands. **k**, *dally*^{ΔP-188} heterozygous embryo derived from an *sgl*⁽³⁾⁰⁸³¹⁰ heterozygous mother. **l**, Cuticle from an *hs-dally*⁺ embryo, after a heat shock pulse.

Dally expressing cells were fractionated using DEAE-Sepharose chromatography. The protein fraction that was eluted at high salt concentrations contained HA-tagged Dally; on SDS-PAGE, HA-Dally protein ran as a broad band of relative molecular mass greater than that of the core protein, a characteristic of proteoglycans (Fig. 1b, c). HA-Dally bears HS chains on two criteria: it is sensitive to a chemical cleavage reaction specific for N-sulphated glucosamine residues found in HS (Fig. 1b) and also to heparin lyase II, an enzyme that selectively cleaves HS chains (Fig. 1c). Like the vertebrate glypicans, Dally does not bear chondroitin sulphate because it is insensitive to chondroitinase ABC (Fig. 1c).

We also examined wild-type Dally protein expressed in third-instar larvae, using an anti-Dally antibody. Like HA-Dally expressed in S2 cells, Dally appeared on SDS-PAGE gels as a broad band with a molecular mass larger than predicted for the protein core, a consequence of glycosaminoglycan addition. The glycosaminoglycan-modified form of Dally was replaced with a discrete species of lower molecular mass in total protein extracts from *sugarless* (*sgl*) mutant larvae (Fig. 1d), showing that Dally modification requires the activity of *sgl*, a gene encoding a glycosaminoglycan biosynthetic enzyme critical for Wg signalling in the epidermis²⁻⁴. Dally is therefore a cell-surface, HS-modified proteoglycan that requires *sgl* activity for its normal biosynthesis.

To determine the effects of *dally* on wg signalling we first examined the cuticles of *dally* mutant embryos. Wg affects the fate of epidermal cells that secrete the larval cuticle, and is required for the formation of the 'naked' cuticle that separates rows of denticles (tooth-like cuticle structures). *wg*-null mutant embryos show a replacement of naked cuticle with denticles along the length of the ventral surface (Fig. 2a, b). Partial loss of *wg* function produces cuticles with reductions in naked cuticle, and a segment polarity defect (Fig. 2c). *dally* mutant embryos show a loss of naked cuticle and abnormalities with segment polarity, phenotypes consistent with a reduction in Wg signalling (Fig. 2d; compare Fig. 2g, h). We have determined the penetrance of cuticle defects in three different allelic combinations of *dally* mutants: *dally*^{ΔP-527}/*dally*^{P2} (21% penetrance, *N* = 608), *dally*^{gem}/*dally*^{ΔP-188} (16% penetrance, *N* = 1,556) and *dally*^{ΔP-188}/*dally*^{ΔP-188} (6% penetrance, *N* = 1,160).

All of the *dally* alleles isolated so far are hypomorphic, and attempts to obtain null mutants as heterozygotes have not been successful (H.N. and S.B.S., unpublished observations). A set of more than 20 deletions in the *dally* gene generated by excision of a P-element insert did not remove the initiating methionine codon, although deletions to within less than 100 base pairs (bp) in the 5' direction were recovered (H.N. and S.B.S., unpublished observations). Furthermore, a deficiency in this region, *Df(3L)h-i22* (ref. 8), is a *dally* hypomorph, with less severe phenotypes than existing *dally* alleles that do not abolish *dally* mRNA expression (data not shown). Our genetic mapping of this deficiency with cytologically defined P-element lethals shows its proximal breakpoint to be between 66D14-15 and 66E1-2, the cytological position of *dally* (*Df(3L)h-i22* fails to complement *l(3)10631* (66D14-15), but complements *l(3)06264* (66E1-2) and *l(3)10534* (66E4)). These findings indicate that *dally* is haplolethal, precluding us from determining to what extent *dally* influences epidermal differentiation by using the existing set of *dally* alleles. We therefore used three other strategies to further explore the role of *dally* in wg signalling: RNA interference (RNAi), genetic interactions between *dally* and *wg* pathway components, and the effects of ectopic Dally expression on Wg signalling.

To characterize further the phenotypes associated with loss of *dally* function we used RNAi, a method established for studies of *Caenorhabditis elegans*⁹ but recently applied to the analysis of *Drosophila* embryogenesis^{7,10}. Embryos injected with *dally* double-stranded RNA alone showed cuticle phenotypes found in *wg* mutants including ectopic denticles, and denticle bands with

polarity defects (Fig. 2f, i, j). The effects of *dally* RNAi were predominantly localized, like those described for RNAi of *wg* and *fz* plus *Dfz2* (of 192 scored embryos, 64 gave denticle belt fusions or ectopic denticle phenotypes)⁷. Buffer-injected embryos did not show these abnormalities and embryos injected with *fushi tarazu* (*ftz*) double-stranded RNA displayed the characteristic phenotype of *ftz* null mutants (Fig. 2e). The phenotypes associated with RNAi of *dally* indicate that *dally* is a critical component of the Wg signalling pathway.

To establish further the role of *dally* in events controlled by Wg we conducted a series of genetic interaction experiments. Genetic interactions have been used extensively to establish functional relationships between genes affecting a common signalling pathway. We examined the genetic interactions between *dally* and *wg* in several ways. First, we determined whether reductions in *dally* function affect the cuticle phenotypes found in a temperature-sensitive *wg* mutant grown at a semi-permissive temperature. The presence of a *dally* hypomorphic allele increased the penetrance of cuticle defects threefold under these conditions of partial *wg* activity (135 abnormal embryos from 775 scored, compared with 41 abnormal from 776 scored). Second, we examined the severity and penetrance of cuticle phenotypes in *dally* homozygous embryos when heterozygous for null mutations in *wg*. Heterozygosity for *wg*-null mutations produced *dally* embryos with more severe phenotypes and at a 2–3-fold higher penetrance. For example, heterozygosity for *wg*^{IL114} increased the penetrance of cuticle defects in *dally*^{ΔP-188}/*dally*^{gem} from 16% to 41% (*N* = 1,045). We observed similar effects with other *wg* and *dally* alleles. These genetic inter-

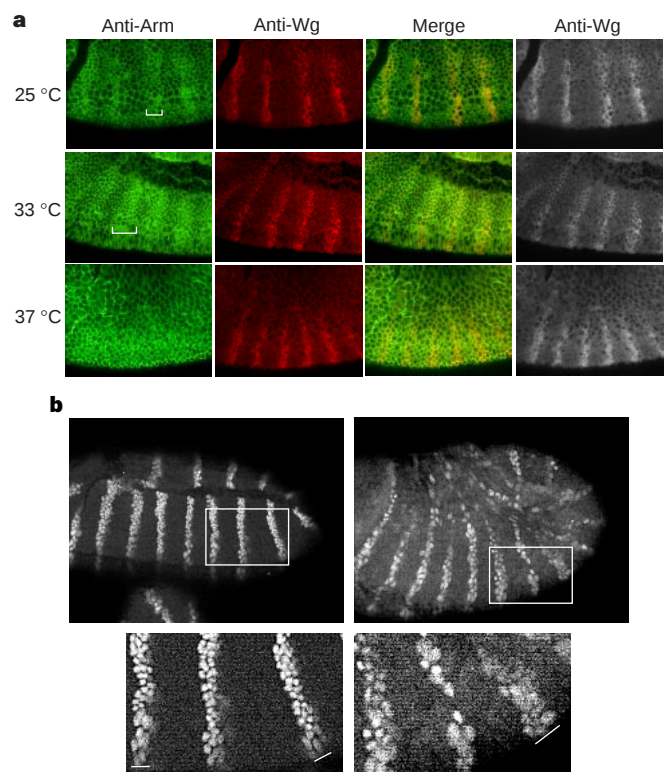


Figure 3 Effects of ectopic Dally expression on cellular responses to Wg. **a**, Anti-Armadillo (Arm) staining of *hs-dally*⁺-bearing embryos (green). Cells with anti-Arm cytoplasmic staining (a marker for Wg signalling) are indicated with brackets in animals treated at 25 and 33 °C. Animals were reared at 25 °C, or with heat pulses at 33 or 37 °C. Anti-Wg staining (red and greyscale images). **b**, Influence of ectopic Dally on expression of En. Wild-type (left) and *hs-dally*⁺ (right) embryos after heat shock stained with anti-En antibodies (side-by-side panels are at the same magnification). *hs-dally*⁺ embryos show enlarged nuclei compared to the wild type. Rectangles mark regions shown in the lower panels. Bars in the lower panels mark the widths of the En stripes in parasegments 5 and 7.

actions indicate that *dally* affects the patterning outcomes directed by Wg signalling in the epidermis.

We also tested the genetic interactions between *dally* and another gene required for normal Wg responses in the epidermis, *sgl* (refs 2–4), which encodes a protein with extensive homology to a vertebrate UDP-glucose dehydrogenase¹¹ (UDPG-DH). The biosynthesis of HS requires UDP-glucuronate, the product of UDPG-DH activity, as a sugar donor for the assembly of the disaccharide polymer. If HS modification of Dally is important for its participation in Wg signalling, reductions in *sgl* function would be expected to enhance *dally* mutant cuticle defects. We confirmed this: embryos heterozygous for *dally* and derived from females heterozygous for *sgl* (*sgl* function is required maternally) showed cuticle defects characteristic of a reduction in Wg signalling (Fig. 2k). Of 554 embryos from *sgl*¹⁽³⁾⁰⁸³¹⁰/*TM3* females crossed with *dally*^{ΔP-188}/*TM3* males, 58 showed a *wg*-like cuticle defect. We observed the same cuticle defects with two independently derived alleles of *dally* and *sgl*, *dally*^{8em} and *sgl*^{N71}. These phenotypes were not found in either *dally* heterozygotes (0/402) or in *sgl*/+ progeny of *sgl*-heterozygous mothers (0/197). The ability of modest reductions in *sgl* function to uncover a dominant phenotype in *dally*-heterozygous embryos indicates strongly that *dally* requires sugar modification for its activity in cuticle patterning. Furthermore, the enhancement of *dally* cuticle defects by reductions in either *wg* or a gene affecting Wg responses, *sgl*, shows that *dally* participates in Wg signalling.

We also examined the ability of ectopic Dally expression to potentiate Wg signalling, in both wild-type embryos and *wg* mutants. Ectopic activation of *wg* signalling throughout the embryonic epidermis directs all cells to secrete naked cuticle¹². Ectopic expression of *dally*⁺ from a *hs-dally*⁺ transgene produces expansion of naked cuticle with a commensurate loss of denticles, which is consistent with an increase in the level of Wg activity (Fig. 2l). We observed similar effects with combinations of UAS-*dally*⁺ (UAS, upstream activating sequence) and different *GAL4* lines, including *en-GAL4* and *prd-GAL4*. These constructs bear the transcription control regions of the *engrailed* and *paired* *Drosophila* genes, respectively (data not shown). However, a high level of *dally*⁺ expression is lethal and few embryos survive to form cuticle. We therefore examined the effects of ectopic *dally*⁺ expression on an earlier stage in development, using antibodies against both Armadillo (Arm) and Wg. Arm is a component of the Wg signalling pathway that, on activation, is stabilized and becomes cytoplasmically localized¹³. We treated *hs-dally*⁺ embryos with brief heat pulses at 33 and 37 °C, and determined the domains of Wg signalling by monitoring Arm immunostaining. Simultaneously we detected Wg protein to determine whether the level of the secreted growth factor was altered (Fig. 3a). In embryos treated with a 33 °C heat pulse, the domains of Arm activation were broader than in the 25 °C reared controls, and a 37 °C treatment produced cytoplasmic localization of Arm in most epidermal cells. However, the pattern of Wg immunostaining did not change in response to ectopic Dally expression.

We also examined the effect of ectopic Dally expression on Engrailed (En), a target of Wg signalling in the epidermis^{14,15} (Fig. 3b). Ectopic expression of Dally did not increase the number of cells that express En in each parasegment, but the animals possessed abnormally large cells, many with condensed nuclei, hallmarks of disrupted cell division. It is therefore likely that the number of cells expressing En was not increased by ectopic Dally as a consequence of disrupted cell division. Yet the physical dimension of some parasegment domains of En expression did expand in animals with ectopic Dally (Fig. 3b, lower panels), suggesting that En is being activated by Dally but this effect is 'masked' by a cell division arrest.

The experiments with *hs-dally*⁺-bearing embryos show that ectopic Dally potentiates Wg-signalling without altering the levels of Wg itself. Therefore, Dally is able to influence cellular responses to Wg, which is consistent with a role as a component of a Wg receptor. These findings also indicate that ectopic *dally*⁺ does not

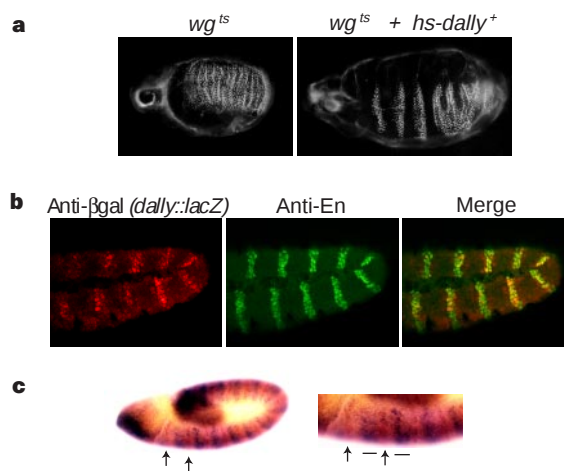


Figure 4 Rescue of *wg* partial loss-of-function mutants by ectopic *dally*⁺ expression and expression patterns of *dally*⁺. **a**, Ectopic expression of *dally*⁺ from an *hs-dally*⁺ transgene rescues a *wg*^{IL144} (a temperature-sensitive allele) embryo reared at 23.5 °C. Left, *wg*^{IL144} embryo; right, a *wg*^{IL144}; *hs-dally*⁺ embryo reared at the same temperature. **b**, Confocal images of a stage 9 embryo bearing a *dally* enhancer trap insert, *dally*^{P22}, stained with anti-βgal antibody (red) and anti-En antibody (green). **c**, *In situ* detection of *wg* and *dally* mRNA in stage 9 embryos (*wg*, red, marked with arrows, *dally*, blue, marked with bars in the right panel). Anterior is to the left, dorsal up. The right panel shows an enlargement of the segments behind the cephalic fold.

influence Wg signalling via Hedgehog, a secreted factor that activates the transcription of *wg*^{16,17}.

We further tested the ability of Dally to potentiate Wg activity by determining whether ectopic Dally expression can rescue a partial loss-of-function *wg* mutant. *wg*^{IL144} is a temperature-sensitive allele that shows reduced Wg secretion at elevated temperatures; at 25 °C, it displays a cuticle defect indistinguishable from that observed in *wg*-nulls^{18,19}. A *hs-dally*⁺ transgene is able to rescue *wg*^{IL144} reared at 23.5 °C; head structures and patterning of the abdominal segment cuticle are restored (Fig. 4a). These animals received no heat pulse, and animals bearing *hs-dally*⁺ showed normal cuticles when reared under these conditions. Animals bearing *wg*^{IL144} are not rescued by the *hs-dally*⁺ transgene at 25 °C, the non-permissive temperature. *hs-dally*⁺ is likewise unable to rescue a *wg*-null allele, *wg*^{CX4}, showing that the ability of *dally* to affect cuticle patterning depends on some remaining Wg activity (data not shown). Thus, ectopic Dally can potentiate Wg signalling but this effect is dependent on some Wg activity remaining at the cell surface. *dally*⁺ does not affect the Wg signalling pathway via some mechanism independent of Wg.

We examined *dally* expression in the embryonic epidermis to determine its relationship with Wg-directed events. *wg* is expressed in segmentally repeated stripes that are two or three cells wide and immediately anterior to cells expressing En. *wg* activity is required for the maintenance of En and Wg expression, showing that *wg*-expressing cells and their immediate neighbours, the En-expressing cells, are responsive to Wg^{14,15,18}. *wg* also affects the differentiation of cells posterior to the En row, because these cells generate different types of denticles, and this denticle diversity requires *wg* activity²⁰. Three independent *dally* enhancer trap lines show a pattern of expression that overlaps with En, indicating that transcription of *dally* occurs in the cells immediately adjacent to the *wg*-expressing cells (Fig. 4b). However, *dally* mRNA is found at the highest levels two or three cell dimensions posterior to *wg*-expressing cells, in contrast with the enhancer trap lines (Fig. 4c). Thus, in epidermal cells exposed to the highest levels of Wg, *dally* mRNA levels are low. The difference between *dally* enhancer trap expression and *dally* mRNA levels suggests that *dally* expression might be regulated post-transcriptionally by Wg signalling.

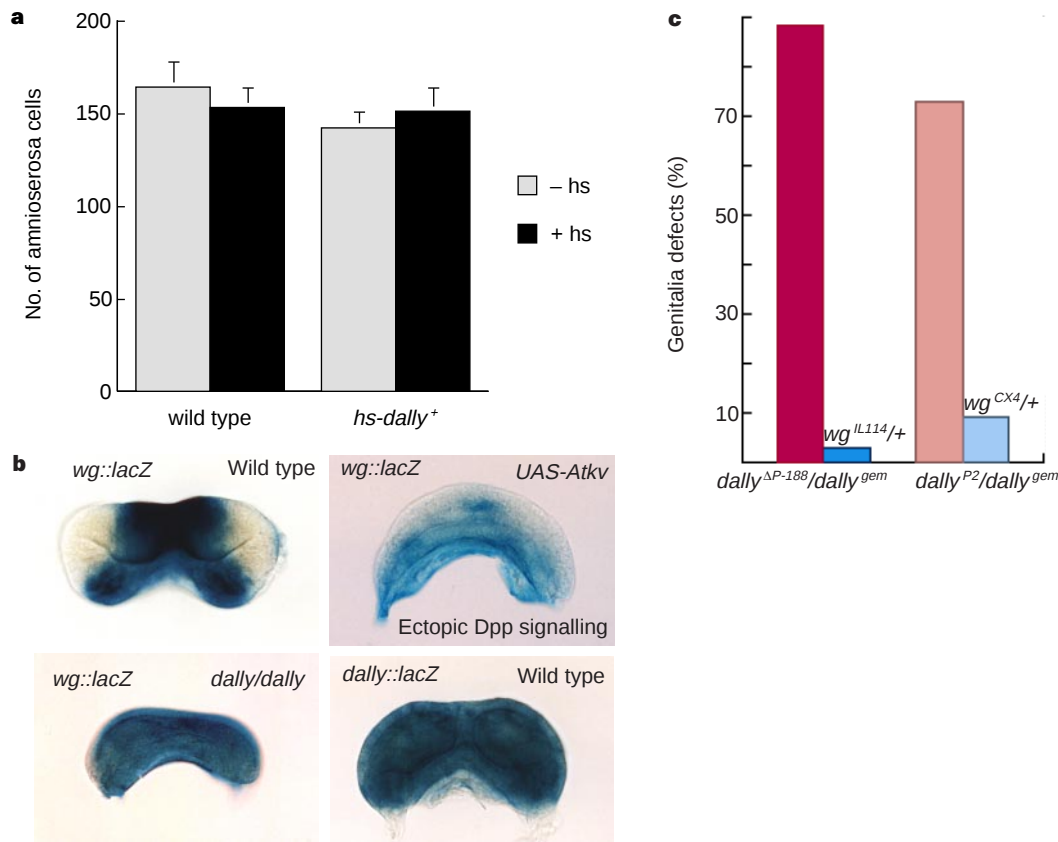


Figure 5 Selective participation of *dally* in Dpp signalling during genitalia development. **a**, Ectopic expression of Dally does not affect Dpp signalling in the embryo, as assayed by the number of amnioserosa cells (wild type, $N = 156$; *hs-dally*⁺, $N = 219$). **b**, *wg* expression (*wg::lacZ*) is repressed by ectopic *dpp* signalling (compare the top two panels). Reductions in *dally* function expand *wg::lacZ*

expression (lower left panel). *dally* enhancer trap expression is found throughout the genitalia disc (lower right panel). **c**, *dally* genitalia phenotypes are repressed by reductions in *wg* function. The percentages of animals with genitalia abnormalities are plotted for *dally* mutants (red and pink bars), and for *dally* mutants heterozygous for either of two *wg* alleles (dark and light blue bars).

Previous studies established that *dally* affects patterning outcomes directed by Dpp during the development of some imaginal tissues⁶. In the embryonic epidermis *dally* influences cellular responses to Wg. To determine whether Dally provides tissue-specific regulation of responses to Wg and Dpp, we examined *dally*-mediated signalling in embryos and imaginal tissue in more detail.

We used the *hs-dally*⁺ transgene to express Dally during early embryonic development, when Dpp is required for the formation of the amnioserosa, a dorsal structure sensitive to the levels of Dpp activity. Incremental changes in Dpp signalling are reflected in the number of amnioserosa cells²¹. Ectopic expression of Dally had no effect on the number of amnioserosa cells, indicating that patterning by Dpp was not potentiated by increasing Dally (Fig. 5a). In contrast, modest levels of ectopic Dally can affect Wg signalling in the epidermis. Dally is therefore preferentially able to affect Wg signalling in the embryonic epidermis.

dally affects patterning directed by Dpp in several imaginal tissues including the genitalia⁶. *dally* mutants have a highly penetrant genitalia defect, which is enhanced by reductions in *dpp* function. *dpp* and *wg* are mutually antagonistic in patterning the genitalia²². The ability of *dpp* signalling to repress *wg* expression is shown by the effects of ectopic expression of a constitutively activated Dpp receptor, Thickvein (*Atkv*), on *wg::lacZ* activity in the third instar genitalia disc (Fig. 5b). If *dally* strictly influences *dpp* signalling during genitalia formation, *dally* mutants should show increased *wg* expression, because of the compromised *dpp* signalling. This is what we observed (Fig. 5b). Expression of a *wg* enhancer trap line is greatly expanded in *dally* mutant discs. *dally::lacZ* expression is found throughout the genitalia disc, suggesting that the selective

participation of *dally* in Dpp signalling is not a consequence of its limited expression.

If *dally* selectively influences *dpp* signalling in the developing genitalia, reductions in *wg* activity should rescue the genitalia abnormalities of *dally* mutants. Two independent mutations in *wg* show a marked, dominant rescue of *dally* genitalia defects (Fig. 5c). *dally* is therefore an antagonist of *wg* function and a promotor of Dpp signalling in genitalia development. Thus, our findings support a selective effect of Dally on cellular responses to Wg in the embryonic epidermis, and Dpp in the developing genitalia. Given the ability of Dally to participate in both Dpp and Wg signalling in a tissue-specific fashion, what might control the selectivity of Dally activity? It might be that Dally bears tissue-specific forms of HS, with the different sugar structures attached to the protein core dictating which growth factor Dally influences. There is great structural diversity in glycosaminoglycan chains, and it seems likely that this diversity provides a means of regulating growth factor signalling at the cell surface²³.

These findings establish that a cell-surface proteoglycan, Dally, participates in Wg signalling. What is the molecular mechanism of Dally's activity? Several lines of evidence support a role for Dally as a regulatory component of the Wg receptor, forming a multiprotein complex with one or more members of the Frizzled (*Fz*) family of transmembrane proteins. First, Wg has a high affinity for heparin/HS²⁴, indicating that Wg is likely to bind HS-modified proteoglycans on the cell surface. Second, cell-surface HS is required for Wg responses in a tissue culture cell line¹. Third, the phenotypes of *sgl* mutants confirm that, *in vivo*, one or more proteoglycans are essential for normal levels of Wg signalling. The fact that the removal of both *fz* and *Dfz2* is required to affect Wg signalling^{7,25,26}

indicates that more than one Fz family member might be assembled into a single signalling complex. Compromising *dally* function either with the hypomorphic alleles available, or by using RNAi, produces phenotypes seen only when both *fz* and *Dfz2* functions are inhibited. Thus, a fully functional Wg receptor in the embryonic epidermis is probably a multiprotein complex that includes one or more Fz proteins and Dally. □

Methods

Expression of epitope-tagged Dally protein. An HA epitope (CYPDVP-DYASL) coding sequence was inserted in frame into an *AatII* site of the *dally* cDNA at amino-acid position 50 (*HA-dally*), and *HA-dally* was cloned into an expression vector (F449) bearing an *hsp70* promoter (*hs-HA-dally*). The *hs-HA-dally* construct was transformed into *Drosophila* S-2 tissue culture cells with standard procedures and HA-Dally expression was induced by a 30 min heat pulse at 37 °C. Cells were pelleted and solubilized in SDS gel sample buffer, and proteins were separated by SDS-PAGE. Dally protein was detected by western blotting on PVDF membranes (Millipore) using anti-HA antibody, HA.11 (Berkley Antibody Co.)

Chemical and enzymatic characterization of HA-Dally. A proteoglycan-enriched fraction containing HA-Dally was obtained from transformed S-2 cells as described²⁷. After ion-exchange chromatography on DEAE-Sephacel (Amersham-Pharmacia), material eluted with 1 M NaCl was dialysed against 0.02 M NH₄HCO₃ and freeze-dried. The dried eluate was dissolved in 0.1 M Tris-HCl, pH 7.2, and subjected to glycosaminoglycan lyase digestions with chondroitinase ABC lyase (EC 4.2.2.4) (chondroitinase, Sigma) and heparin lyase II (Sigma) as described²⁷. After digestion, the samples were analysed by SDS-PAGE and western blotting. For low-pH nitrous acid treatments of proteoglycan-enriched material from HA-Dally-expressing S2 cells, aliquots of partly purified proteoglycans were digested as described²⁸, and detected by SDS-PAGE and western blotting with anti-HA antibody.

For PI-PLC (Sigma) treatment, cells (2×10^6 cells per reaction) were suspended in 200 µl of 50 mM Tris-HCl, pH 7.6. The cell suspension was treated with 1 U of PI-PLC (EC 3.1.4.10, Sigma) at 37 °C for 2 h; the reaction was stopped by adding 400 µl of 0.1 M Na₂CO₃, pH 11.5. After 30 min on ice, soluble and insoluble (membrane-bound) fractions were separated by centrifugation. Proteins extracted from each fraction were analysed by immunoblotting with anti-HA antibody.

Genetic experiments. Animals were reared at 25 °C unless stated otherwise. The standard embryo collection for preparation of cuticles consisted of a 4-h egg collection, followed by incubation for 20 h at 25 °C. Embryos were fixed and devitelinized cuticles prepared with standard procedures. Penetrance of *dally* embryonic phenotypes were calculated by assuming that one-quarter of embryos derived from *dally*-heterozygous parents would be homozygous for *dally*. For the analysis of the effect of ectopic *dally*⁺ on cuticle patterning, *hs-dally*⁺-bearing embryos were given a 60-min heat pulse at 37 °C, 3 h after egg-laying. *UAS-dally*⁺ and *hs-dally*⁺ constructs are described in ref. 6. Ectopic expression of constitutively activated Thickvein (Tkv) in the genitalia disc was accomplished with a *ptc-GAL4* line, together with *UAS-ca-tnk*. *wg* expression was monitored with a *wg::lacZ* reporter, *wg^{17en40}*. RNAi experiments were conducted as described⁷. Double-stranded RNA was synthesized from a plasmid bearing a full-length *dally* cDNA clone (*c1*)⁵.

The studies on the influence of ectopic *dally*⁺ expression on Arm and Wg expression used two heat-shock regimes. Embryos were collected for 2 h at 25 °C and allowed to develop for 2 h further. The embryos were then collected into phosphate buffer with 0.1% Tween, and placed at either 33 or 37 °C for 20 min. Embryos were returned to 25 °C on an agar plate and left to develop for 2 h, then fixed and processed for immunofluorescence. Antibody staining of embryos was performed as described²⁹ (anti-Wg (1:200)⁵, anti-En (mouse monoclonal used at 1:1 dilution), anti-Arm (polyclonal N2 at 1:500), and rabbit anti-βgal (Cappel, preabsorbed against fixed embryos, then used at 1:5,000)).

For the analysis of the effects of ectopic *dally*⁺ expression on aminioserosa cell development, *hs-dally*⁺ embryos were treated with a 15–20-min heat pulse at either 30 or 33 °C at the cellular blastoderm stage (2 h 30 min to 3 h 15 min after egg-laying). Stage 10 embryos were collected and stained with anti-Kruppel antibody (1:300). Methyl salicylate was used to clear the embryos; the

number of aminioserosa cells was counted with differential interference contrast (DIC) microscopy.

wg and *dally* mRNA were detected in whole-mount embryos by using digoxigenin-UTP and fluorescein-UTP-labelled RNA probes.

Generation of anti-Dally antibodies. A *XhoI*–*BamHI* fragment of the *dally* cDNA was subcloned into the expression vector pQE31 (Qiagen), producing a truncated Dally protein fused in frame with a polyhistidine tag. Dally expression was induced with isopropylthiogalactoside and cells were collected by centrifugation. After cell lysis, recombinant Dally was purified by chromatography on a Ni-nitrilotriacetate column (Qiagen), emulsified with adjuvant (TiterMax; CytRx. Co.), and injected subcutaneously into rabbits. After three boosts at 10-day intervals serum was harvested and used at 1:500 dilution for western blotting of total protein from wild-type and *sgl* mutant crawling third-instar larvae.

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Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling

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The *Drosophila* wingless gene (*wg*) encodes a protein of the Wnt family and is a critical regulator in many developmental processes¹. Biochemical studies have indicated that heparan sulphate proteoglycans, consisting of a protein core to which heparan sulphate glycosaminoglycans are attached², are important for Wg function³. Here we show that, consistent with these findings, the *Drosophila* gene *sulfateless* (*sfl*), which encodes a homologue of vertebrate heparan sulphate *N*-deacetylase/*N*-sulphotransferase (an enzyme needed for the modification of heparan sulphate) is essential for Wg signalling. We have identified the product of *division abnormally delayed* (*dally*), a glycosyl-phosphatidyl inositol (GPI)-linked glypican, as a heparan sulphate proteoglycan molecule involved in Wg signalling. Our results indicate that Dally may act as a co-receptor for Wg, and that Dally, together with *Drosophila* Frizzled 2, modulates both short- and long-range activities of Wg.

Wg signalling is defective in *sugarless* (*sgl*) mutants⁴. *sgl* encodes a *Drosophila* homologue of uridine diphosphoglucose dehydrogenase that is required for the formation of glucuronic acid. Because glucuronic acid is required for the formation of heparan sulphate, chondroitin sulphate and dermatan sulphate, it is unclear which classes of proteoglycans are involved in Wg signalling. In the genetic screen⁵ that led to the isolation of *sgl*, we also isolated mutations in a second locus *sulfateless* (*sfl*), that showed a similar segment-polarity cuticle phenotype (Fig. 1b). In *sfl* null embryos, the expression patterns of *wg* and *engrailed* (*en*) are reminiscent of those observed in either *wg* or *hedgehog* (*hh*) null mutants (not shown). To investigate further whether Sfl activity is required in Wg signalling, we analysed the effect of *sfl* mutations on the development of stomatogastric nervous system (SNS) and the second midgut

constriction, both of which require Wg but not Hh activity⁶. In *sfl* null embryos, the development of these Wg-mediated processes is perturbed (Fig. 1e, g). Consistent with a role for Sfl in Wg signalling, Wg-dependent processes in the wing imaginal disc also require Sfl activity. Wg is required for dorso-ventral patterning and acts over a short range to control the expression of *neuralized* (*neu*) at the wing margin¹ and in the long range to activate the expression of *distalless* (*dll*)^{7,8}. In *sfl*-mutant wing discs, the expression of *neu* is abolished (Fig. 1i), and Dll expression is also markedly reduced (Fig. 1k). Our results indicate that Sfl activity is necessary for Wg signalling during both embryonic and wing-disc development.

A complementary DNA encoding the product of *sfl* was isolated (Fig. 2). A search of the protein sequence databases revealed that the putative protein deduced from the *sfl* cDNA is homologous with heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST)⁹, which is required specifically for the modification of heparan sulphate glycosaminoglycans (GAGs) but not chondroitin sulphate and dermatan sulphate GAGs. Together, these results provide genetic evidence that heparan sulphate proteoglycans (HSPGs) are involved in Wg signalling and that HSPGs have non-redundant roles with other classes of proteoglycan in the context of Wg signalling.

Heparan sulphate GAGs are attached to various protein cores to form different HSPGs. Because *Drosophila* frizzled 2 (*Dfz2*) appears as a distinct band on western blots (S. Cumberledge, personal communication; M. Zeidler and N.P., unpublished), not as the smear that is characteristic of proteoglycans², it is unlikely that the receptors for Wg/Wnt encoded by members of the Frizzled (Fz) family are heparan-sulphate-modified proteins. The *Drosophila* glypican homologue *dally* appeared to be an excellent candidate because flies homozygous for hypomorphic *dally* alleles exhibit some wing-margin defects¹⁰, a phenotype similar to partial loss of *wg* activity. To examine the role of *dally* in Wg signalling, we first determined the expression of *dally* messenger RNAs in embryos by *in situ* hybridization (Fig. 3). At early stages *dally* transcripts are uniformly expressed; however, at stage 8, *dally* transcripts are enriched in a segmental repeated pattern in three to four cells anterior to *wg*-expressing cells. Double staining for *Dfz2* mRNA and *wg*-lacZ shows that the 2–3-cell-wide band of *dally*-expressing cells anterior to *wg*-expressing cells also express *Dfz2*¹¹ (not shown), indicating that *dally* may be involved in Wg signalling.

Next, we examined the cuticle phenotype of *dally* mutant

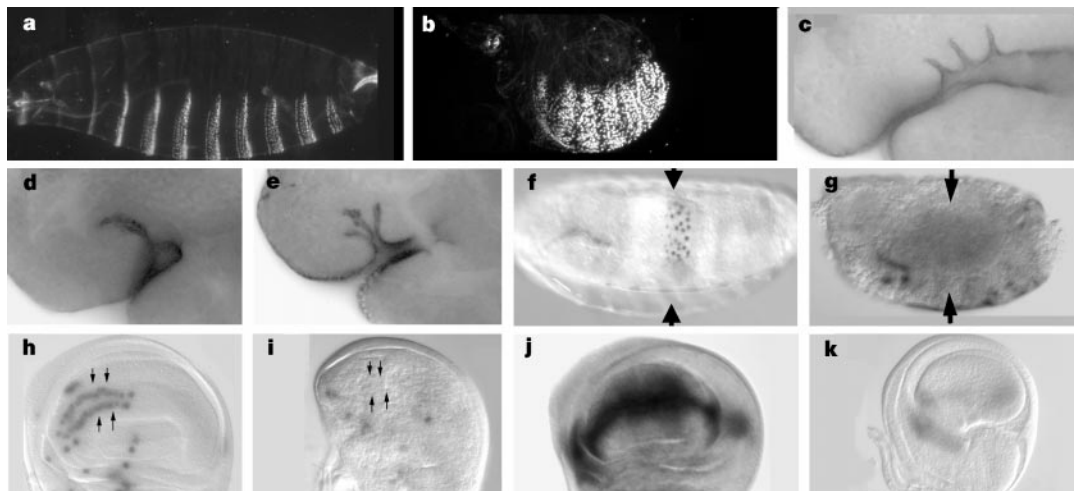


Figure 1 *sfl* is required for Wg signalling. **a, b**, Cuticle phenotypes of wild-type (WT) (**a**) and *sfl* (**b**) embryos. **c–e**, SNS phenotypes stained by anti-Crumbs antibody in stage 10 embryos of WT (**c**), *wg* (**d**), *sfl* (**e**). As observed for *sgl*⁶, the SNS phenotype is similar, although slightly weaker, than in the *wg* mutant (ref. 6). **f, g**, The expression of Labial (Lab) in WT (**f**) and *sfl* (**g**) embryos at stage 15; Lab staining, marking the position where the second midgut (arrows) is absent in an

sfl embryo (**g**). In the wing disc of WT third-instar larvae (**h**), *neuralized* (*neu*)-expressing sensory mother cells visualized using the A101 enhancer trap are found in two rows (arrows), and are missing in *sfl*⁽³⁾⁰³⁹⁴⁴/*sfl*^{9B4} wing disc derived from *sfl* homozygous mutant animals derived from heterozygous mothers (**i**). In WT wing disc (**j**), Dll forms a gradient with its highest level of expression at the dorso-ventral boundary, and is greatly reduced in the *sfl*⁽³⁾⁰³⁹⁴⁴/*sfl*^{9B4} disc (**k**).

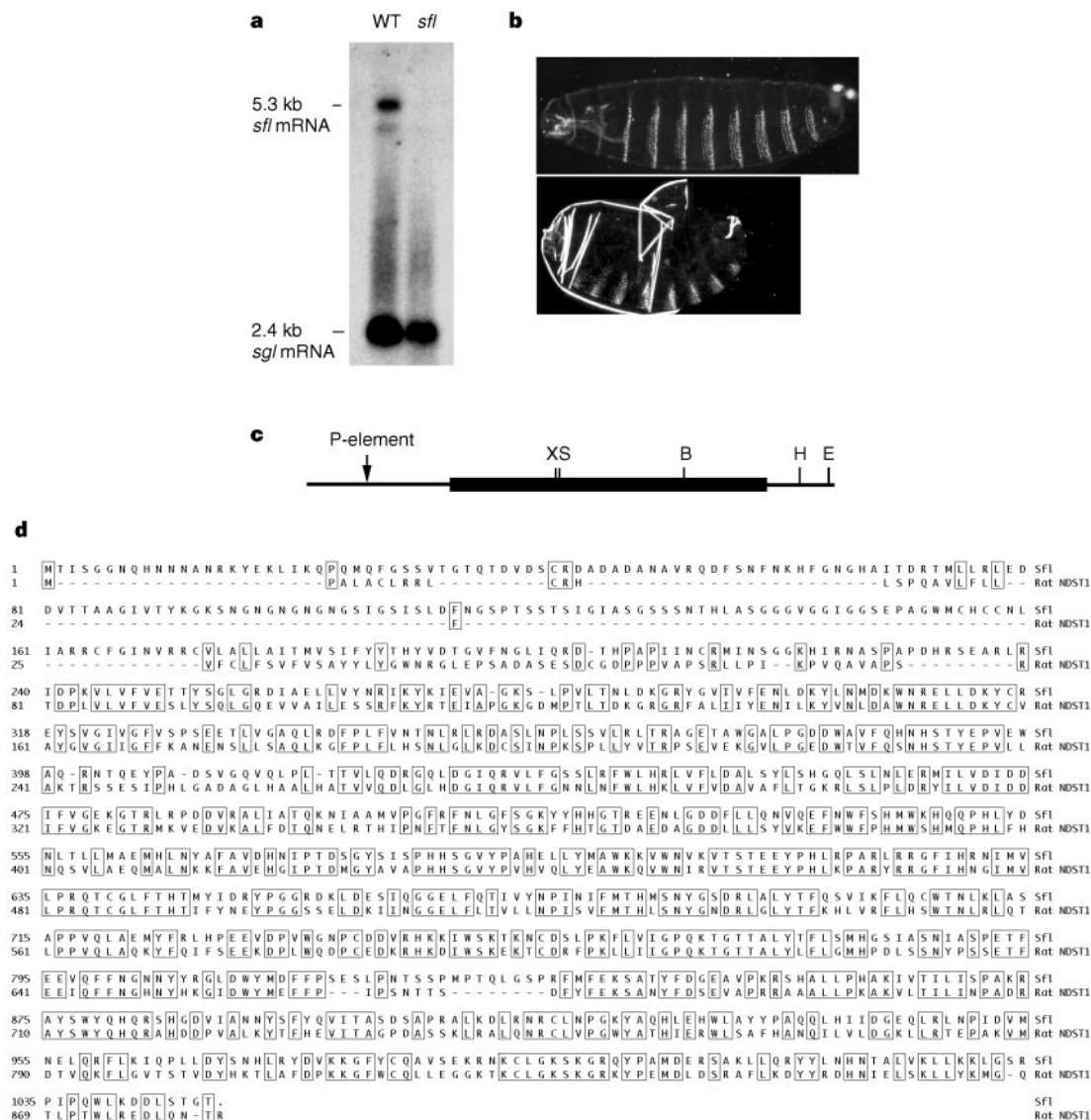


Figure 2 *sfl* encodes heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST). **a**, Northern blot analysis of *sfl* RNA from 0–1.5 h WT and *sfl* mutant embryos. The blot was probed with *sfl* cDNA and *sgl* cDNA⁶. *sgl* was used as an internal control. 5.3 and 2.4 kilobase (kb) mRNAs correspond to the *sfl* and *sgl* transcripts, respectively. **b**, Rescue of *sfl* maternal-effect phenotypes by RNA injection. Top, cuticle phenotype of a paternally rescued *sfl* embryo marked with a *trachealess* (*trh*) mutation that exhibits the defective posterior spiracles⁶. Bottom, cuticle phenotype of *sfl* null embryos derived from GLCs injected with RNA transcribed from the *sfl* full-length cDNA. Of 700 injected embryos derived from

females with *trh* GLCs, 120 *sfl* mutant embryos (*trh sfl/sfl*) developed scorable cuticle structures, and 15% of them showed evidence of rescue. **c**, Restriction map of the *sfl* cDNA. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sst*I; X, *Xho*I. The P-element *l*(3)03844 is inserted at base 576 of the *sfl* cDNA (686 base pairs (bp) upstream of a putative ATG start codon). The open reading frame encoding Sfl is shown by the thick line. **d**, Putative amino-acid sequence of Sfl protein and comparison with rat NDST1. Identical residues are boxed. The overall identity between Sfl and Rat NDST1 is 51%.

embryos. These mutant embryos exhibit poorly penetrant cuticle segment-polarity defects resembling a partial defect in Wg signalling (Fig. 3d). However, these segmentation defects can be significantly enhanced by removal of one copy of *sfl* in the mother or one copy of *wg* in the embryo (Fig. 3e, f). Because all available *dally* mutations are weak alleles, we used double-stranded RNA (dsRNA) interference to block *dally* gene activity¹². Embryos injected with *dally* dsRNAs corresponding to the entire coding region of *dally* exhibit severe segment-polarity cuticle defects (Fig. 3g, h), similar to those injected with *wg* or *frizzled* (*fz*) + *Dfz2* dsRNAs¹². This result, together with the genetic interaction observed between *sfl* and *wg*, strongly supports the proposal that *dally* is a new segment-polarity gene and that it is required for Wg signalling in the embryo. Further, we find that Dally, which migrates as a smear in wild-type extracts, migrates as sharp bands in the protein extracts of *sfl* mutants (Fig. 4). Similarly, Dally is not modified in *sgl* embryos (S. Selleck, personal

communication). These results indicate that Dally is a likely substrate of Sgl and Sfl.

To further examine the role of *dally* in Wg signalling, we analysed the function of *dally* during wing-disc development. Consistent with previous reports¹⁰, we found that only 3% of homozygous *dally* animals exhibit wing-margin defects (Fig. 5a). This frequency can be increased 2–3-fold and wing-margin defects are more severe when one copy of *wg* is removed (Fig. 5b). To determine whether Dally cooperates with the Wg receptor Dfz2 in wing patterning, we tested whether *dally* mutations can enhance a loss-of-function *Dfz2* phenotype. When a dominant-negative form of *Dfz2* (*Dfz2N*)¹³ is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects (Fig. 5c). However, this phenotype is enhanced in homozygous *dally* mutants (Fig. 5d), indicating that *dally* may potentiate Wg signalling. Furthermore, ectopic expression of a gain-of-function

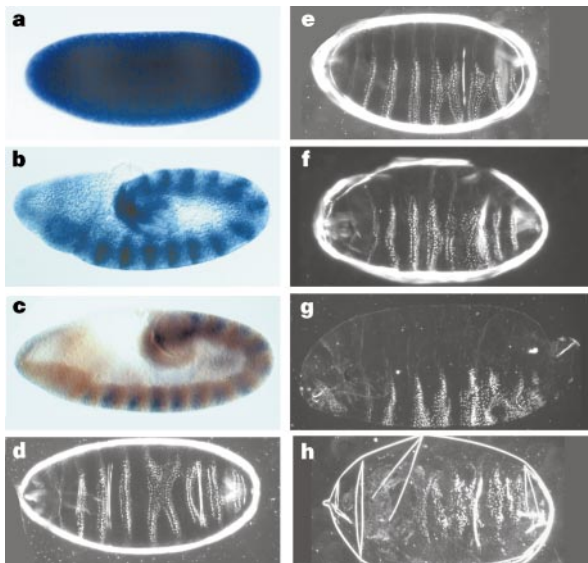


Figure 3 *dally* is a segment-polarity gene. *dally* mRNAs are uniformly distributed in stage 2 embryos (**a**), and are expressed in a segmental repeated pattern at stage 8 (**b**). **c**, *dally* transcripts (blue) are located 3–4 cells anterior to *wg*-expressing cells (brown, *wg-lacZ*). *dally*^{P2} homozygous embryos derived from females with *dally*^{P2} or *dally*^{AP-188} homozygous GLCs exhibit weak segment-polarity cuticle defects (**d**; 8% penetrance, *n* = 760). More severe defects are detected in *dally*^{P2} embryos where *sfl* maternal message is eliminated (**e**; 14% penetrance; *n* = 780). Similarly, *dally*^{P2} homozygous embryos derived from GLCs show a more severe embryonic phenotype if zygotic *wg* is reduced by half (**f**; 16% penetrance, *n* = 760). Embryos injected with *dally* dsRNA develop *wg*-like cuticle defects (**g**, **h**). 48% of the injected embryos (*n* = 127) exhibit defects. Embryos injected with buffer exhibit no cuticle defects (*n* = 150).

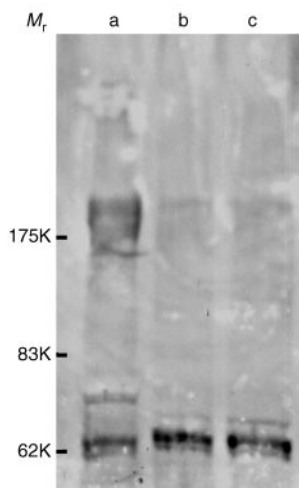


Figure 4 Heparan sulphate GAG modification of Dally in *sfl* mutants. Total proteins from third-instar larvae were analysed by SDS-PAGE followed by western blotting with anti-Dally antibody. In WT larvae (**a**), Dally migrates as a high relative molecular mass (*M_r*) smear, characteristic of heparan sulphate-modified Dally, and ~70K unmodified bands. In homozygous *sfl*⁽²⁾⁰³⁸⁴⁴ (**b**) or *sfl*^{9B4} (**c**) larvae, high *M_r* heparan sulphate-modified Dally is significantly reduced and sharp bands of unmodified Dally are increased.

Arm protein (*Arm*^{act}) can fully rescue the wing defects (Fig. 5f), indicating that the enhancement of wing-margin defects in the *dally* mutant is specific to Wg signalling and that Dally acts upstream of Arm. These genetic interactions are consistent with a role for Dally in Wg signalling and indicate that Dally may act with Dfz2 in Wg reception.

If *dally* acts with Dfz2 to transduce Wg signalling, Dally may also

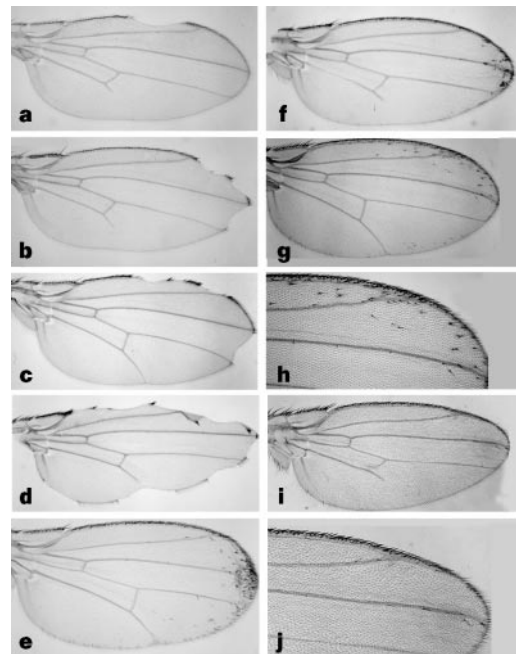


Figure 5 Dally is required for Wg/Dfz2 signalling in wing patterning. **a**, 3% of *dally*^{P2} homozygous flies exhibit a wing notching phenotype (*n* = 550). **b**, Wing phenotype of *wg*^{IG22}/+; *dally*^{P2}/*dally*^{P2} animals is enhanced and shows higher penetrance (8%; *n* = 654). The wing-vein abnormality seen in the *dally* mutant does not seem to involve Wg signalling. **c**, Ectopic expression of Dfz2N at the presumptive wing margin using C96-Gal4 (*UAS-Dfz2N*/+; *C96*/+) is associated with a fully penetrant mild wing-margin defect. **d**, *UAS-Dfz2N*/+; *C96 dally*^{P2}/*dally*^{P2} wing. Decreased *dally* activity strongly enhances the wing defect observed in **c**. **e**, *UAS-arm*^{act}/+; *C96*/+ wing. Ectopic expression of *arm*^{act} results in ectopic bristles on the wing blade. **f**, *UAS-arm*^{act}/*UAS-Dfz2N*; *C96 dally*^{P2}/*dally*^{P2} wing. Ectopic expression of *UAS-arm*^{act} fully rescues the margin defect shown in **d**. **g**, **h**, Uniform expression of Dfz2 driven by 69B-Gal4 in *69B-Gal4/UAS-Dfz2* flies leads to wings with ectopic bristles. **i**, **j**, Ectopic bristles are strikingly reduced in the wing of *UAS-Dfz2 dally*^{P2}/*69B-Gal4 dally*^{P2}. **h**, **j**, Higher magnifications of the wings shown in **g** and **i**, respectively.

be required for other functions of Dfz2 in Wg signalling. In the wing blade, Dfz2 is involved in shaping the gradient of Wg distribution and determining the response of cells to Wg¹⁴. Uniform overexpression of *Dfz2* in the wing pouch leads to ectopic bristle formation in the wing blade, probably reflecting activation of Wg signalling above its normal level. Ectopic expression of *Dfz2* driven by the Gal4 line 69B resulted in wings with ectopic bristles¹⁴ (Fig. 5g, h). In a *dally* mutant background, the formation of ectopic bristles was greatly reduced, indicating that a mutation in *dally* blocks the activity of Dfz2 (Fig. 5i, j).

Our findings indicate that HSPGs have non-redundant roles with other classes of proteoglycan in Wg signalling, and that *dally* encodes a protein core of the HSPGs involved in Wg signalling. There are several possible mechanisms for the function of Dally in Wg signalling. First, Dally could form an active Wg receptor complex with Dfz2. Second, Dally, through its heparan sulphate GAG sequences, could generate a higher-affinity binding site for Wg to Dfz2. Third, as proposed for other co-receptors¹⁵, Dally could limit the free diffusion of Wg by capturing it on the cell surface, thereby increasing its local concentration and the probability that it will interact with less abundant, high-affinity signalling receptors. Biochemical analyses between Dally, Wg and Dfz2 will be required to distinguish between these models. Interestingly, both *Dfz2* and *Fz* encode redundant Wg receptors in the embryo¹². Thus it is possible that, in addition to having a role in the Wg/Dfz2 interaction, Dally also cooperates with Wg/Fz. Furthermore, Dally regulates the

activity of decapentaplegic (Dpp)¹⁶, a member of the TGF- β superfamily. As we have no evidence for a role for HSPGs in the early function of Dpp in the establishment of dorso-ventral embryonic polarity, the function of Dally may be tissue-specific. Tissue-specific effects of Dally could be generated either through tissue-specific expression of *dally* during development or tissue-specific modification of the heparan sulphate GAG chains linked to the Dally protein core. There is biochemical and genetic evidence to support the model that specific heparan sulphate GAGs decorate the cell surface. In vertebrates, a number of sulphotransferases are differentially expressed in various tissues¹⁷. In addition, the *Drosophila* gene *pipe*, which is involved in dorso-ventral patterning in the embryo, encodes a putative heparan sulphate 2-O sulphotransferase that is expressed in ventral follicle cells¹⁸. □

Methods

Reagents. The *sfl* alleles are *sfl*⁽³⁾⁰³⁸⁴⁴ (ref. 5) and *sfl*^{9B4} (N.P., unpublished). Both *sfl*⁽³⁾⁰³⁸⁴⁴ and *sfl*^{9B4} show similar maternal-effect phenotypes. Females with germline clones (GLCs) were generated as described⁶. All the available *dally* alleles are homozygous viable to some extent, with *dally*^{p2} and *dally*^{ΔP188} representing the strongest alleles available¹⁰. To try to isolate a stronger loss-of-function *dally* allele, we generated a number of new *dally* alleles by P-element excisions. However, none was stronger than the original¹⁰. Other stocks are: UAS-Dfz2 (ref. 14) and UAS-Dfz2N (ref. 13), UAS-arm^{act} (ref. 19), C96 Gal4 (ref. 20). *dally* cDNA was obtained from S. Selleck¹⁰. Crumbs, Dll and Lab antibodies were obtained from E. Knust, I. Duncan and T. Kaufman, respectively.

Molecular methods. Molecular characterization of *sfl* and RNA injection were done as described for *sgf*⁶. Western blotting of Dally was performed using a polyclonal Dally antibody, a gift of H. Nakato. The dsRNA synthesis and injection were as described¹².

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The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins

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The regulation of gene expression depends critically upon chromatin structure¹. Transcription of protein-coding genes can be reconstituted on naked DNA with only the general transcription factors and RNA polymerase II (ref. 2). This minimal system cannot transcribe DNA packaged into chromatin, indicating that accessory factors may facilitate access to DNA. Two classes of accessory factor, ATP-dependent chromatin-remodelling enzymes³ and histone acetyltransferases⁴, facilitate transcription initiation from chromatin templates. FACT (for facilitates chromatin transcription) is a chromatin-specific elongation factor required for transcription of chromatin templates *in vitro*^{5,6}. Here we show that FACT comprises a new human homologue of the *Saccharomyces cerevisiae* Spt16/Cdc68 protein and the high-mobility group-1-like protein structure-specific recognition protein-1. Yeast *SPT16/CDC68* is an essential gene that has been

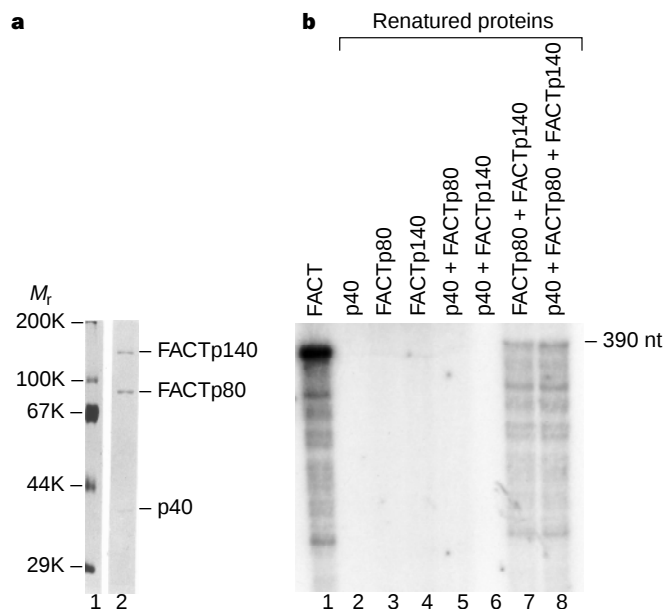


Figure 1 Recovery of FACT activity from renatured p140 and p80 subunits. **a**, Silver-stained gel (phosphocellulose column, fraction 9) showing proteins used in FACT renaturation experiment. The positions of FACTp140, FACTp80, the p40 protein and protein relative molecular mass (M_r) markers are indicated. **b**, Mixtures of renatured polypeptides assayed for FACT activity. Polypeptides were renatured alone and in all possible combinations and were used in transcription reactions on remodelled chromatin templates containing Gal4-VP16 and a reconstituted transcription system (lanes 2–8). Purified FACT was used in lane 1. FACT activity was measured by the appearance of long RNA molecules. The position of the full-length, 390-nucleotide RNA product is indicated.

implicated in transcription and cell-cycle regulation. Consistent with our biochemical analysis of FACT, we provide evidence that Spt16/Cdc68 is involved in transcript elongation *in vivo*. Moreover, FACT specifically interacts with nucleosomes and histone H2A/H2B dimers, indicating that it may work by promoting nucleosome disassembly upon transcription. In support of this model, we show that FACT activity is abrogated by covalently crosslinking nucleosomal histones.

FACT appears to be a heterodimer of subunits of relative molecular mass (M_r) 140K and 80K⁵. To determine whether these subunits are responsible for FACT activity, the polypeptides from the most purified FACT fraction (Fig. 1a, lane 2) were eluted from a polyacrylamide-SDS gel, denatured and renatured individually or in combination. FACT activity required both p140 and p80 polypeptides (~5% recovery of activity; Fig. 1b, lane 7). A third, 40K polypeptide present in our FACT preparations (Fig. 1a), but not co-eluting with FACT activity⁵ (p40; Fig. 2a) was not required for activity (Fig. 1b, lane 7), could not reconstitute activity in combination with FACTp140 or FACTp80 alone (Fig. 1b, lanes 5 and 6) and had no effect on FACT activity in the presence of both FACTp140 and FACTp80 (Fig. 1b, lane 8). We conclude that p140 and p80 are both necessary and sufficient for FACT activity.

Using peptide sequences from FACTp140 obtained by ion trap mass spectrometry, we isolated three overlapping complementary DNA clones that together encode a 1,047-amino-acid-protein with a relative molecular mass of 119,900 (119.9K). The open reading frame contained the sequences of all peptides obtained, indicating that the cDNA encodes FACTp140 (see Supplementary Information). Moreover, antibodies generated against a portion of the open reading frame recognized FACTp140 (Fig. 2a). FACTp140 is 36%

identical to *S. cerevisiae* Spt16/Cdc68 (refs 7, 8) (see Supplementary Information), which is an essential nuclear protein required for normal transcription of a wide variety of genes transcribed by RNA polymerase II (RNAP II)⁷⁻⁹. In *spt16* mutants, arrest at the START phase of the cell cycle occurs¹⁰ because of a defect in transcription of the G1 cyclins required for progression through START^{8,11}. These genetic data indicate that FACT is required for transcription *in vivo*.

Although previous evidence is consistent with a role for Spt16 in transcription and chromatin structure *in vivo*, there was no evidence implicating Spt16 in transcription elongation. In light of our biochemical data, we investigated this possibility by determining whether Spt16 interacts genetically with two established transcriptional elongation factors, TFIIS and the Spt4 subunit of the Spt4/5 complex. The *PPR2* gene, which encodes TFIIS, is not essential for cell viability but a *ppr2Δ* deletion causes extreme sensitivity to 6-azauracil (6AU), a phenotype (6AU^S) that is linked specifically to transcription elongation¹². The *PPR2* gene was deleted in related *SPT16*⁺, *spt16-197* and *cdc68-11* strain pairs and the resulting strains were scored for 6AU^S phenotypes (Fig. 3a). Consistent with previous results, the *SPT16 ppr2Δ* strain was sensitive to 200 $\mu\text{g ml}^{-1}$ 6AU, whereas *spt16-197* alone exhibited only a weak 6AU^S phenotype. However, *spt16-197* suppressed the 6AU^S phenotype caused by *ppr2Δ*. This effect is not strain-specific because *cdc68-11*, which encodes the same amino-acid replacement as *spt16-197* in a different genetic background¹³, also suppressed the *ppr2Δ* 6AU^S phenotype. Suppression can be attributed specifically to *cdc68-11*, as the 6AU^S phenotype of *ppr2Δ* was restored by plasmid-borne *SPT16*.

The Spt4/Spt5 complex binds RNAP II and affects elongation both positively and negatively^{14,15}. Like *PPR2*, the *SPT4* gene is not essential for cell viability, but an *spt4Δ* deletion causes 6AU^S.

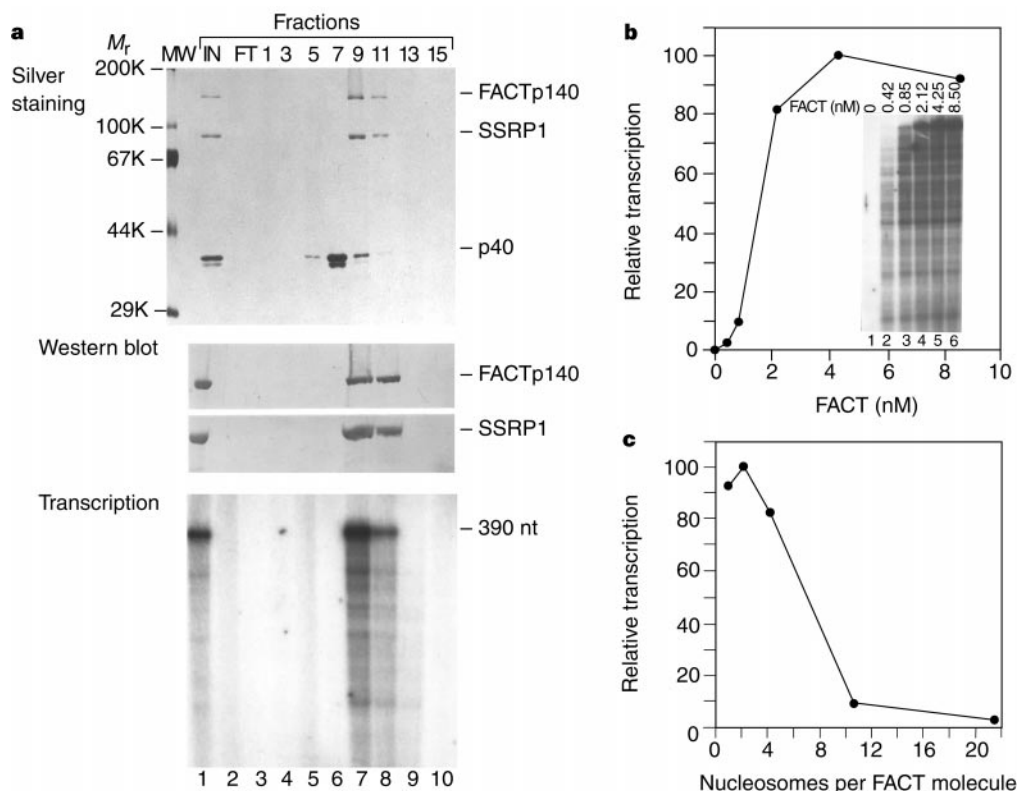


Figure 2 FACT activity co-elutes with FACTp140 and human SSRP1. **a**, Assay of protein fractions from a phosphocellulose column by silver staining (top panel), western blots using anti-FACTp140 and anti-SSRP1 antibodies (middle two panels) and transcription analysis using remodelled chromatin templates containing Gal4-VP16 and a reconstituted transcription system (bottom panel). The positions of FACTp140, SSRP1, M_r markers and the 390-nucleotide RNA product are shown. **b**, Determination of the concentration of FACT required for

maximal stimulation of transcription elongation on chromatin templates. Varying concentrations of FACT were used in transcription reactions with 0.45 nM chromatin template. The transcription experiment is shown as an inset. The amount of full-length, 390-nucleotide RNA is expressed as relative transcription. **c**, Maximal FACT activity requires about one molecule of FACT for every two nucleosomes. The data from **b** are expressed as nucleosomes per FACT molecule assuming a nucleosomal spacing of 170 bp⁵.

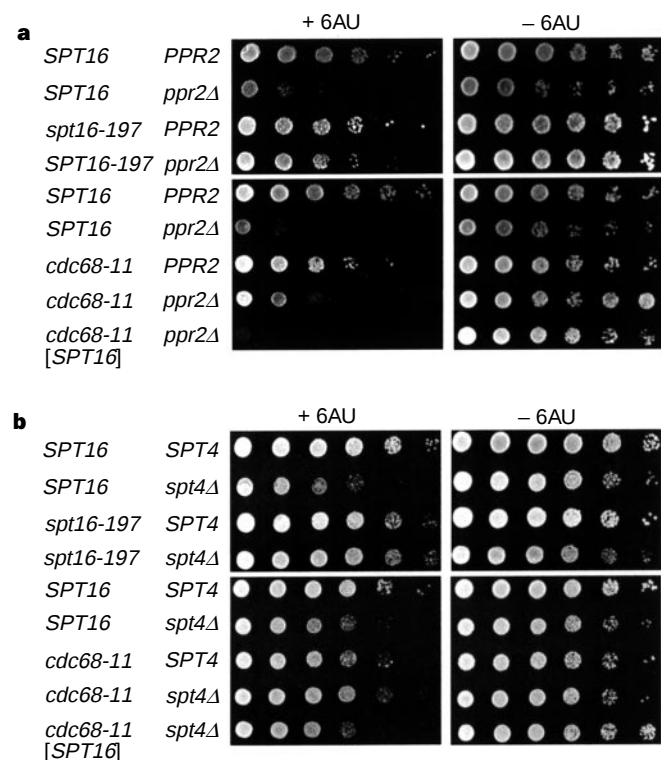


Figure 3 The *spt16-197* and *cdc68-11* alleles suppress the 6AU^S phenotypes associated with the *ppr2Δ* and *spt4Δ* mutations. Tenfold serial dilutions of strains with the indicated genotypes were spotted onto synthetic complete medium either containing or lacking 200 $\mu\text{g ml}^{-1}$ 6AU. Plates were photographed following incubation at 30°C for 2 days. **a**, Interaction of *spt16-197* and *cdc68-11* with *ppr2Δ*. **b**, Interaction of *spt16-197* and *cdc68-11* with *spt4Δ*. For complete genotypes and sources of these strains, see Supplementary Information.

Furthermore, *spt4Δ* enhances the 6AU^S phenotype of a *ppr2Δ* strain¹⁴. In a manner analogous to the *ppr2Δ* experiments, we found that *spt16-197* and *cdc68-11* suppressed the 6AU^S phenotype of *spt4Δ* in two different genetic backgrounds and that this effect was rescued by plasmid-borne *SPT16* (Fig. 3b). These results establish a genetic relationship between Spt16 and the elongation factors TFIIS and Spt4, and provide strong support for involvement of FACT and Spt16 in transcription elongation *in vivo*.

The ability of *spt16-197* and *cdc68-11* to suppress the 6AU^S phenotype associated with *ppr2Δ* and *spt4Δ* seems counter-intuitive. However, there is a precedent for this type of observation. The *rpb2-10* mutation, which encodes an altered form of RNAP II and decreases the rate of elongation *in vitro*¹⁶, suppresses the cold-sensitive growth defect of an *spt5* mutation *in vivo*¹⁴. This result was interpreted to mean that the reduced rate of elongation enables RNAP II to transcribe past potential barriers to elongation when the Spt4/5 complex is impaired. A similar model might explain suppression of *ppr2Δ* and *spt4Δ* by the *spt16* defect. The slower elongation caused by *spt16* would be expected to diminish the frequency of arrest, thereby alleviating the dependence upon TFIIS and Spt4/5.

Peptide sequences from FACTp80 corresponded to sequences found in the human structure-specific recognition protein-1 (SSRP1)^{17,18}. The relative molecular mass of SSRP1 (81K) is consistent with the mobility of FACTp80 by polyacrylamide-SDS gel electrophoresis, and antibodies raised against SSRP1 recognize an 80K protein in FACT preparations that co-elutes with FACT activity (Fig. 2a). We conclude that FACT-80 is SSRP1. The homologue of SSRP1 in *S. cerevisiae* is encoded by the essential *POB3* gene¹⁹. Pob3 is found in an abundant nuclear complex with Spt16 (ref. 20).

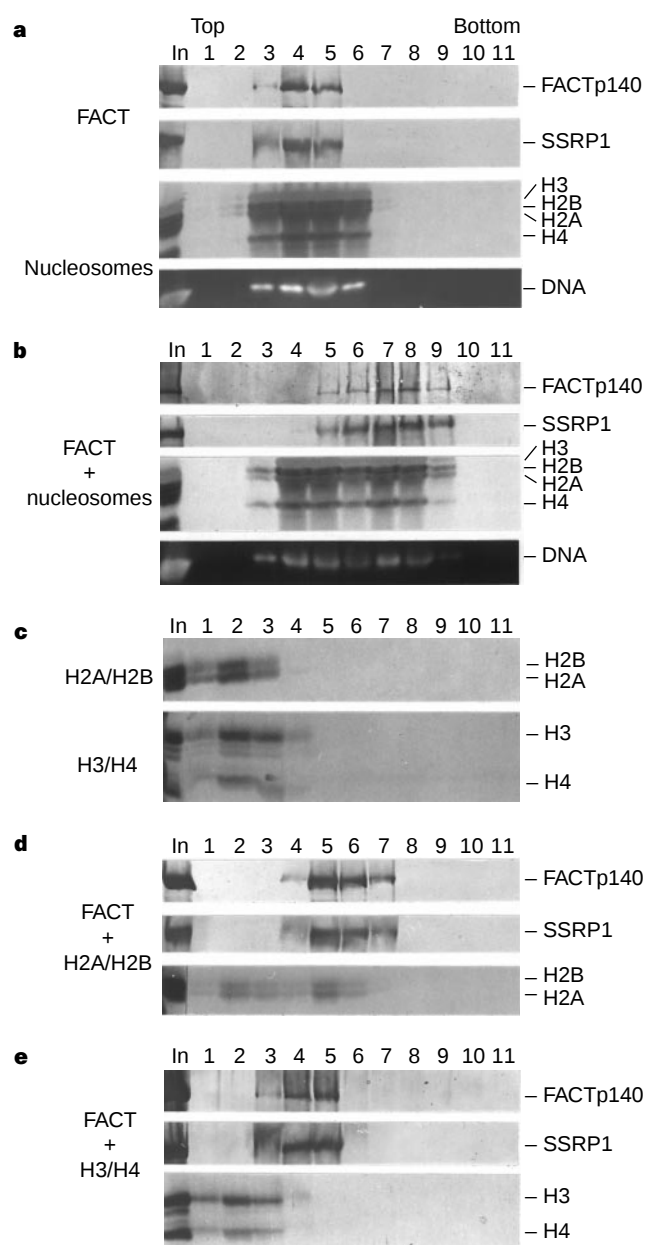


Figure 4 FACT specifically binds to mono-nucleosomes and histone H2A/H2B dimers. FACT alone, mono-nucleosomes alone (**a**), histone H2A/H2B dimer alone, histone H3/H4 tetramer alone (**c**), a mixture of FACT and mono-nucleosomes (**b**), FACT and histone H2A/H2B (**d**) and a mixture of FACT and histones H3/H4 (**e**) were sedimented through 15–50% glycerol gradients. Fractions from the gradients were analysed for FACT by western blotting using anti-FACT-140 and anti-SSRP1 antibodies, for histones by silver staining and for nucleosomal DNA (**a**, **b**) by agarose gel electrophoresis and ethidium-bromide staining.

SSRP1 contains a high-mobility group (HMG)-1 domain. Proteins containing HMG-1 domains bind to DNA at the cross-over point where it enters and exits a nucleosome²¹. Therefore, the HMG-1 domain of SSRP1 may target FACT to nucleosomes to facilitate passage by RNAP II. Consistent with this model, we found that the concentration of FACT required for maximal activity was directly proportional to the concentration of the chromatin template and was independent of the concentration of RNAP II (data not shown). Using 0.45 nM chromatin template, 4.25 nM FACT produced maximal stimulation of elongation (Fig. 2b). This

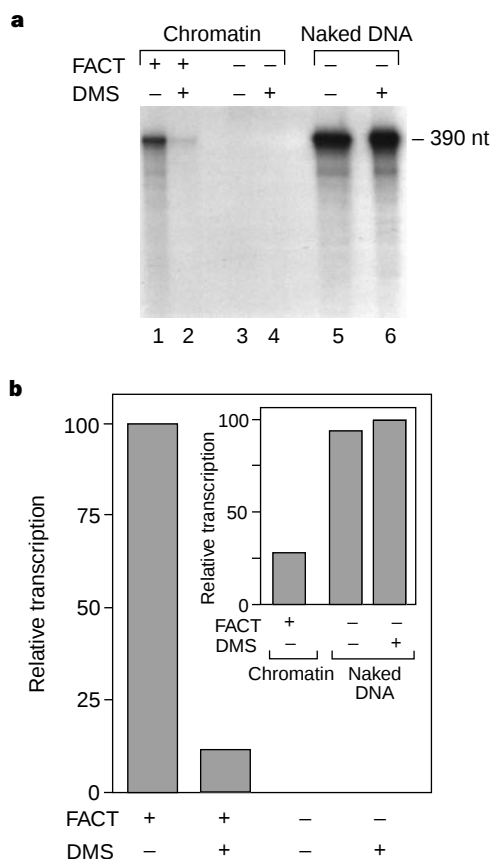


Figure 5 FACT activity requires disruption of the histone octamer. Remodelled chromatin templates were treated with DMS to covalently crosslink nucleosomal histones to prevent dissociation of histones H2A and H2B. Crosslinked templates (**a**, lanes 2 and 4) and control templates (**a**, lanes 1 and 3) were then used in transcription reactions with the additions indicated. To determine whether DMS modifies and inactivates DNA, naked DNA templates subjected to the same treatment in the absence and presence of DMS were used in transcription reactions (**a**, lanes 5 and 6). **b**, Quantification of the relative amounts of 390-nucleotide RNA product measured in the experiment shown in panel **a**. The main graph compares the levels of transcription obtained with crosslinked and control chromatin templates in the absence and presence of FACT. The inset compares the levels of transcription obtained using chromatin templates with those obtained using naked DNA.

corresponds to approximately one molecule of FACT for every two nucleosomes (Fig. 2c). Based on purification yields, we estimate that there are more than 100,000 molecules of FACT per cell (data not shown). This is sufficient for FACT to be targeted to a large proportion of active genes (see Supplementary Information).

To investigate how FACT facilitates transcript elongation, we examined whether it can interact specifically with nucleosomes. FACT was incubated with an excess of mono-nucleosomes (average DNA length 180 base pairs (bp)) and the sedimentation profile of the mixture on a glycerol gradient was analysed and compared to that obtained with FACT alone and mono-nucleosomes alone (Fig. 4a). The addition of FACT to mono-nucleosomes produced a second, faster-sedimenting peak of histones and DNA with a concomitant shift of the FACT peak (Fig. 4b). Thus, FACT forms a stable complex with a nucleosome.

To probe the interaction between FACT and nucleosomes further, we examined whether FACT can interact directly with the isolated histone components of a nucleosome. FACT was incubated with an excess of histone H2A/H2B dimers or H3/H4 tetramers and the sedimentation profiles of the mixtures were analysed and compared

to those obtained with FACT alone (Fig. 4a), H2A/H2B alone and H3/H4 alone (Fig. 4c). The addition of FACT to the H2A/H2B dimer resulted in the appearance of a second, faster-sedimenting peak of H2A/H2B accompanied by a shift of the FACT peak (Fig. 4a, c, d). Thus, FACT stably and specifically interacts with the histone H2A/H2B dimer. By this criterion, FACT did not interact with either the histone H3/H4 tetramer (Fig. 4e) or RNAP II (data not shown).

These results have implications for the mechanism of action of FACT. Nucleosomes containing transcribed sequences are deficient in histones H2A and H2B and are preferentially bound by RNAP II (ref. 22). Moreover, removal of histones H2A and H2B from a nucleosome enhances transcript elongation by RNA polymerase III (ref. 23) and promotes the binding of transcription factors to their sites³. These results indicate that FACT may function by binding to nucleosomes and displacing one or both H2A/H2B dimers upon transcription.

In support of this model, yeast strains with mutations in histone H4 that alter the interface between the H2A/H2B dimer and the H3/H4 tetramer exhibit identical phenotypes to strains carrying mutations in *spt16*, including an *spt* phenotype and arrest at the START phase of the cell cycle²⁴. This finding led to the proposal that Spt16 regulates the H2A/H2B–H3/H4 interaction in a nucleosome²⁴. Moreover, *spt16* mutants share phenotypes with histone H2A and H2B mutants²⁵ and with *spt4*, *spt5* and *spt6* mutants, which affect transcription elongation^{14,15}. This genetic data, together with our biochemical data indicating that FACT is required in near stoichiometry with nucleosomes, and interacts with nucleosomes and the isolated H2A/H2B dimer, indicates that FACT may function by promoting disruption of the histone octamer upon transcription by RNAP II.

To test this model, we examined whether FACT activity requires disruption of the histone octamer. Chromatin templates were treated with dimethyl suberimidate (DMS) to covalently crosslink nucleosomal histones to prevent dissociation of H2A/H2B²⁶. The crosslinked chromatin templates were then used in FACT transcription assays (Fig. 5a). FACT activity on the crosslinked templates was reduced to 11% of the activity on templates subjected to the same treatment without DMS (Fig. 5a, lanes 1 and 2; Fig. 5b). This effect is not due to inactivation of the DNA template by DMS, because identical treatment did not affect the transcription activity of a naked DNA template (Fig. 5a, lanes 5 and 6). The synthesis of long transcripts on chromatin templates requires FACT⁵ (Fig. 5a, lanes 1–4). These data demonstrate that chromatin templates are rendered resistant to the action of FACT by covalently crosslinking nucleosomal histones, and indicate that FACT activity requires dissociation of nucleosomal histones during transcription elongation.

Based on our data, we propose a model for the mechanism of FACT activity. FACT associates with the nucleosomes of a transcribed gene upon transcription activation. When a transcribing RNAP II approaches, FACT may promote disruption of the histone octamer by binding and removing one or both histone H2A/H2B dimers. This leaves only the histone H3/H4 tetramer bound to DNA, which presents a lesser obstacle to RNAP II. Note that binding of histones H2A/H2B is not sufficient for FACT activity, as the histone-binding protein nucleoplasmin does not promote elongation through nucleosomes⁵. The mechanism by which FACT is specifically targeted to the nucleosomes of active genes remains to be determined.

The presence of homologues of FACTp140 and SSRP1 in evolutionary diverse organisms indicates that FACT may be essential for transcription in all eukaryotes. Our results indicate that the critical function of Spt16 and Pob3 in yeast is due, at least in part, to their role in transcript elongation through nucleosomes. It is possible that FACT facilitates other nuclear processes that require access to nucleosomal DNA. Indeed, the finding that the yeast counterpart to

FACT interacts specifically with DNA polymerase α (ref. 19) suggests a role for FACT in DNA replication. The identification of the subunits of FACT reported here should allow the reconstitution of complex transcriptional regulatory systems in a more physiological environment. □

Methods

Renaturation of FACT subunits. We purified FACT from HeLa cells as described⁵. FACT activity was reconstituted from denatured subunits as described²⁷ with modifications (see Supplementary Information). The efficiency of recovery of FACT activity from renatured subunits was ~5%.

In vitro transcription assays. We used transcription assays using a reconstituted human transcription system and remodelled chromatin templates to measure FACT activity as described⁵. To determine the concentration of FACT required for maximal stimulation of transcription elongation through nucleosomes, we used varying concentrations of FACT (0.42–8.5 nM) in reactions containing 0.45 nM plasmid chromatin template.

Protein sequencing. Following in-gel S-carboxyamidomethylation and tryptic digestion, peptide sequence information was determined by micro-capillary reverse-phase chromatography²⁸ coupled to the electrospray ionization (ESI) source of a quadrupole ion trap mass spectrometer (Finnigan LCQ). The ion trap's online data-dependent scans allowed the automatic acquisition of a high resolution scan to determine charge state and exact mass, and MS/MS spectra for peptide sequence information. Identification of spectra corresponding to known peptide sequences in the NCBI nr and dbest databases was facilitated using the algorithm SEQUEST²⁹, followed by manual confirmation.

Isolation of FACTp140 cDNA. Sequences of two peptides obtained from FACTp140 (ICDVYNAVMDVVK and YTEGVQSLNWTG) were encoded by human EST clones 511016 and 650031, respectively. We used *EcoRI* restriction fragments from these ESTs, corresponding to nucleotides 712–990 and 2585–3230 of the FACTp140 cDNA, to screen a HeLa cell cDNA library (Clontech). Three overlapping clones were obtained corresponding to nucleotides –298 to +1061, +940 to +2413 and +2374 to +4648 (where +1 is the first nucleotide of the AUG initiator codon). The complete cDNA encoded a 3,141-bp open reading frame with in-frame stop codons upstream and downstream that was expressed in all tissues examined (data not shown).

Antibodies. A region of the FACTp140 open reading frame corresponding to amino-acids 276–572 was subcloned into plasmid pET28a (Novagen) to produce plasmid pET28a–FACTp140_{276–572} for protein expression in *Escherichia coli*. Protein purification using Talon affinity resin (Clontech), production of polyclonal antibodies and western blots were performed using standard methods.

Glycerol gradient sedimentation. We purified histone dimers and tetramers to apparent homogeneity from HeLa cells. To prepare mono-nucleosomes, oligonucleosomes purified from HeLa cells were mildly digested with Micrococcal nuclease (Sigma) to produce mono-nucleosomes of average DNA length 180 bp. These were purified further by sucrose gradient centrifugation. We incubated mixtures of FACT (20 μ g) and histone H2A/H2B dimers (5 μ g), FACT and histone H3/H4 tetramers (10 μ g) and FACT and mono-nucleosomes (containing 20 μ g histones) in Buffer G (20 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.4 mM PMSF, 10 mM β -mercaptoethanol and 5% glycerol) for 5 min at 25 °C followed by 30 min at 0 °C. We applied the mixtures (200 μ l) to 15–50% glycerol gradients in buffer G (5 ml) and centrifuged them for 17.5 h at 45,000g. Fractions (400 μ l) were analysed for FACT subunits, histones and DNA.

Crosslinking of nucleosomal histones with DMS. Purified remodelled chromatin templates or naked DNA (1.6 μ g) were dialysed against 0.1 M sodium borate (pH 10), 0.1 M NaCl, 0.1 mM EDTA, and bovine serum albumin (Gibco BRL) was added to a final concentration of 0.2 mg ml^{–1}. DMS (2 mg ml^{–1}; Pierce) was added to covalently crosslink nucleosomal histones²⁶ and the reactions were incubated for 45 min at 25 °C. The reactions were then dialysed for 90 min against 25 mM Tris-HCl (pH 6.8), 1 mM EDTA and for 90 min against 10 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. FACT transcription assays were performed as described⁵, except that Gal4–VP16 (25 nM) was added to compensate for the loss of activator during the crosslinking procedure. Control reactions were subjected to an identical procedure without DMS.

Yeast genetic analysis. The *prr2* Δ and *spt4* Δ mutants are isogenic to the indicated *SPT16/CDC68*, *spt16-197* and *cdc68-11* strains and were constructed by one-step gene disruption using either the *XhoI*–*PvuII* DNA fragment (*prr2* Δ ::*hisG*–*URA3*–*hisG*) of plasmid pJD3 or an *spt4*::*HIS3* DNA fragment obtained by PCR amplification of the *HIS3* gene with synthetic *spt4*–*his3* hybrid oligonucleotides oPH183 and oPH184.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Millisecond-timescale motions contribute to the function of the bacterial response regulator protein Spo0F

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Protein backbones and side chains display varying degrees of flexibility, which allows many slightly different but related conformational substates to occur¹. Such fluctuations are known to differ in both timescale and magnitude, from rotation of methyl groups (nanoseconds) to the flipping of buried tyrosine rings (seconds)^{2,3}. Because many mechanisms for protein function require conformational change, it has been proposed that some of these ground-state fluctuations are related to protein function⁴. But exactly which aspects of motion are functionally relevant remains to be determined. Only a few examples so far exist where function can be correlated to structural fluctuations with known magnitude and timescale^{5,6}. As part of an investigation of the

mechanism of action of the *Bacillus subtilis* response regulator Spo0F, we have explored the relationship between the motional characteristics and protein–protein interactions. Here we use a set of nuclear magnetic resonance ¹⁵N relaxation measurements to determine the relative timescales of Spo0F backbone fluctuations on the picosecond-to-millisecond timescale. We show that regions having motion on the millisecond timescale correlate with residues and surfaces that are known to be critical for protein–protein interactions.

The *B. subtilis* response regulator Spo0F is pivotal in the bacterial signal transduction pathway that controls the morphological change from a vegetative cell to a dormant endospore⁷. It is activated by phosphorylation and performs its function through protein–protein interactions with members of three different protein families: histidine protein kinases, phosphatases and a phosphotransferase protein. Studies using alanine scanning mutagenesis^{8,9} indicate that Spo0F uses a single, semicontiguous surface for its interactions with members of each of these families. The specific members are KinA (kinase), RapB (phosphatase) and Spo0B (phosphotransferase). Preliminary studies of backbone dynamics indicate that Spo0F has flexibility on this surface¹⁰. To characterize the correlation between Spo0F motions and its protein–protein interactions more accurately, we combined results from a nuclear magnetic resonance (NMR) ¹⁵N relaxation study with the information obtained from the study of alanine scanning mutagenesis. The resulting data provide one of the strongest correlations so far between protein dynamic fluctuations on the microsecond-to-millisecond timescale and function.

The following NMR relaxation parameters were determined for 108 of Spo0F's 124 backbone amide nitrogens: rate constants

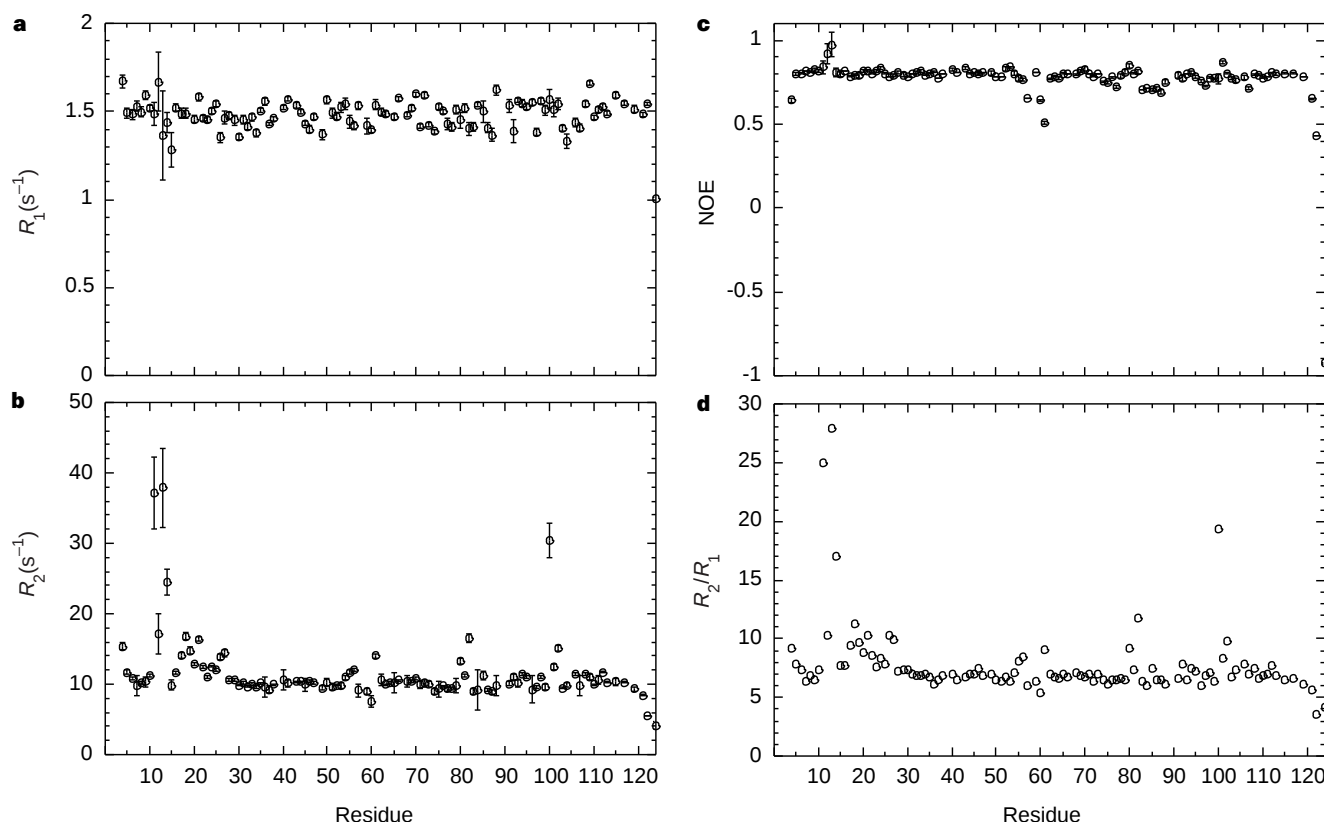


Figure 1 Determination of NMR relaxation parameters. NMR relaxation R_1 (a) and R_2 (b) rate constants and NOE values (c) could be determined for 108 of the 116 detected Spo0F ¹⁵N nuclei observed in a ¹⁵N HSQC spectrum. Residues not included are A39, I42, K67, M89, A114, K116 and L118, owing to severe spectral

overlap and line-broadening of I90. The unobservable amides, three N-terminal residues, the C-terminal penultimate residue (S123) and four prolines account for the total 124 Spo0F residues. d, R_2/R_1 ratios.

for spin–lattice relaxation (R_1); spin–spin relaxation (R_2); and steady-state ^1H – ^{15}N nuclear Overhauser effects (NOE) (Fig. 1a–c). The mean values (10% trimmed) are $1.49 \pm 0.02 \text{ s}^{-1}$, $10.6 \pm 0.5 \text{ s}^{-1}$, 0.79 ± 0.01 and $6.86 \pm 0.05 \text{ ns}$ for the R_1 , R_2 , steady-state NOE and R_2/R_1 , respectively. These values (Fig. 1d) are similar to those recorded previously¹⁰ for Spo0F ($2.2 \pm 0.2 \text{ s}^{-1}$, $10.0 \pm 0.5 \text{ s}^{-1}$, 0.78 ± 0.1 and $4.5 \pm 0.4 \text{ ns}$, respectively). The differences in R_1 and R_2 averages can be attributed to the difference in the magnetic field strength of 500 MHz compared with 600 MHz. Fig. 1d illustrates that the overall trend for the R_2/R_1 ratios is the same as previously measured and the overall fit of the order parameters has little difference for secondary structure (0.89 ± 0.01 compared with 0.09 ± 0.05 reported previously). Large differences are observed in the NOE values for residues in loop regions, which arise from saturation transfer errors in the NOE experiments reported in ref. 10. Excluding the $\beta 1$ – $\alpha 1$ loop, all of our reported NOE values fall below the theoretical maximum¹¹.

The mean values of R_1 , R_2 and steady-state NOE provided an initial estimate of overall correlation time (τ_m) of $7.72 \pm 0.3 \text{ ns}$. This value of τ_m and the other relaxation parameters were fit to five extended Lipari–Szabo dynamic models¹² based on the methodology described in ref. 13. The models considered were: (1) S^2 only, τ_e and R_{ex} are negligible; (2) S^2 and τ_e only, R_{ex} is negligible; (3) model 1 and an R_{ex} term; (4) model 2 and an R_{ex} term; and (5) incorporation of an additional order parameter for anisotropic rotational diffusion¹⁴. The relative fits provided estimates, for each N–H vector, for order parameters (S^2), correlation times of fast internal motion for motions on the timescale greater than 20 ps (τ_e) and a chemical exchange term for slow timescale motions on the order of microseconds to milliseconds (R_{ex}) (Fig. 2a–c).

Generally, the order parameters indicate that regions of secondary structure behave rigidly on the picosecond-to-nanosecond timescale (Fig. 2a). The loop regions and the carboxy terminus have lower order parameters indicating fast motion in addition to the fast tumbling experienced by the entire protein. Residues that required τ_e or R_{ex} terms (Fig. 2b, c respectively) to describe their relaxation parameters are mapped onto the average solution structure of apo-Spo0F (Fig. 3a, b respectively) to outline structural correlations to dynamic regions. Regions experiencing fast internal perturbations (τ_e values greater than 100 ps) are isolated to the extreme amino and C termini and to several residues in a loop immediately following the site of phosphorylation (I57, M60 and D61). Other residues in this loop have statistically insignificant values of τ_e , but their relaxation parameters fit best to models 2 or 5, indicating that the entire loop may be experiencing a cooperative fast timescale fluctuation. Residues with R_{ex} values greater than 1 s^{-1} are experiencing fluctuations on the microsecond-to-millisecond timescale and cluster in several regions: (1) the loop between β -strand 1 and α -helix 1 (termed the $\beta 1$ – $\alpha 1$ loop) and α -helix 1; (2) residues at the ends of the $\beta 3$ – $\alpha 3$ loop; (3) the $\beta 4$ – $\alpha 4$ loop and N-terminal end of α -helix 4; and (4) the β -strand 5 and $\beta 5$ – $\alpha 5$ loop. Residues I17, H101, K104 and F106 do not fit any of the models, indicating that additional contributions to their relaxation are not accounted for. All are found in regions with R_{ex} and may experience multiple timescale fluctuations. There are strong correlations between regions of Spo0F with high backbone deviations and lower $S_{ang}(\phi, \psi)$ and residues having τ_e and R_{ex} fluctuations contributing to the ^{15}N relaxation¹². These regions correspond to the loops of $\beta 1$ – $\alpha 1$, $\beta 3$ – $\alpha 3$, $\beta 4$ – $\alpha 4$, $\beta 5$ – $\alpha 5$ and α -helix 4 and β -strand 5. Therefore the dynamics data confirm that the structural deviations observed in the NMR ensemble of structures are not artefacts. Many residues experiencing R_{ex} fluctuations cluster in specific areas, indicating that concerted motions may be giving rise to the ^{15}N relaxation characteristics observed.

It is essential to exclude non-functional factors that could contribute to the relaxation. Small amounts of rotational anisotropy may contribute to R_{ex} (ref. 15). For Spo0F there is a potential

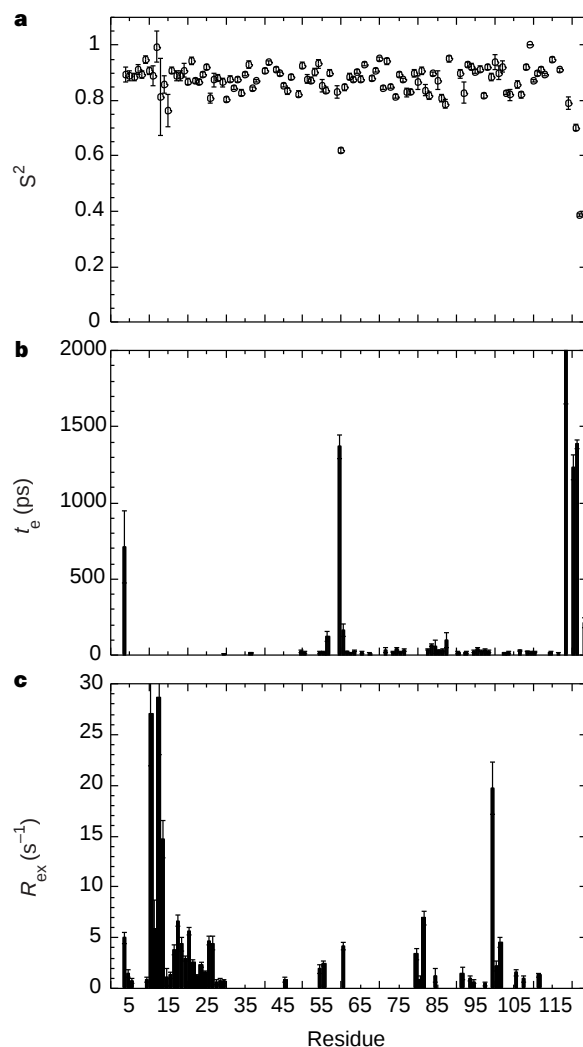


Figure 2 Results from Modelfree analysis are plotted according to primary sequence position. **a**, S^2 ; **b**, τ_e ; **c**, R_{ex} . Of the 108 nuclei subjected to the analysis, 29 were best fit by model 1, 19 by model 2, 32 by model 3, 5 by model 4 and 7 by model 5. Residues E4, I17, I76, H101, K104, F106 and N124 did not fit any of the models; models 5, 3, 2, 3, 3 and 4 were used for final calculations. The SSE (sum of square errors) residuals were not higher than 5.0 (mean, 1.37) for the residues that were fit by models 1–3. For residues that did not fit the simple model-free formalism in the previous study¹⁰, residues D11 to G14, R16 to G27, T82, K94, T100 and F102 are consistent with model 3, which contains a slow-timescale conformational exchange term. Other residues, namely G59, K70 and G85, are most consistent with models 1, 1 and 4, respectively.

anisotropic contribution from α -helix 1, which displays relatively large R_{ex} terms. The solution structure of Spo0F, R_2/R_1 values and appropriate methods¹⁶ were used to fit relaxation rate constants using rotationally asymmetric or anisotropic tumbling models. The $D_{||}/D_{\perp}$ asymmetric rotational diffusion ratio of 1.07 ± 0.02 indicates negligible overall anisotropy and data were not better fit to either anisotropic model when analysed by an F -test of χ^2 values (data not shown). The maximum artefactual R_{ex} contribution was calculated to be 1.04 s^{-1} . In addition, α -helix 1 does not align with the slowest rotational axis. These data indicate that Spo0F tumbles isotropically. Protein aggregation was ruled out by studies of dynamic light scattering and the reproducibility of ^1H – ^{15}N heteronuclear single quantum coherence (HSQC) line-widths and chemical shifts throughout data collection. Thermal denaturation effects were excluded by a study of circular dichroism

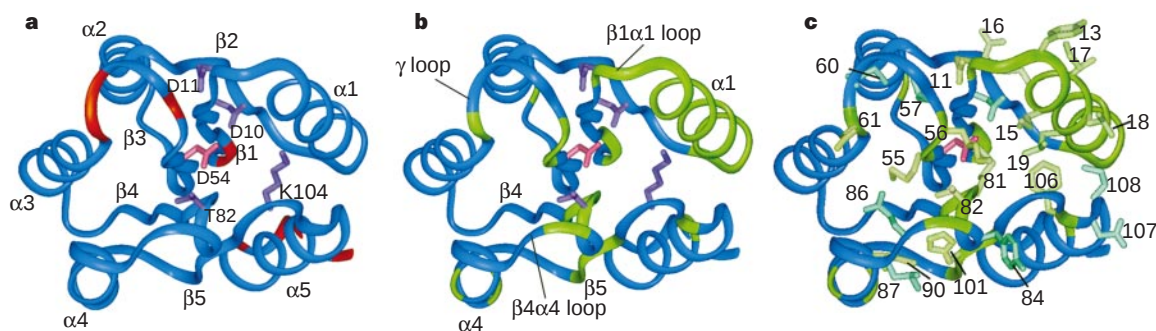


Figure 3 Correlating protein motions with protein-protein interactions. **a**, A ribbon illustration of the Spo0F solution structure (Protein Data Bank code: 2fsp (ref. 10)) highlights the site of phosphorylation at D54 (pink) and residues that are essential for phosphorylation and activity: D10, D11, T82 and K104 (lavender). Backbone amides that have significant internal motion, τ_e , are indicated (red). **b**, Amides that have significant motion on a microsecond-to-millisecond timescale, R_{ex} , are indicated on the Spo0F backbone ribbon (green). **c**, Correlation of motion on the R_{ex} timescale and Spo0F function; all residues exhibiting altered sporulation phenotypes when mutated to alanine are illustrated (side chains shown in green

and light blue) and backbone regions shown to have conformational exchange on the microsecond-to-millisecond timescale are the same as **b**. Residues D11, Y13, I15, R16, L18, L19, M55, K56, M81, T82, I90, H101 and F106 correlate directly with residues that have significant R_{ex} terms; I57, M60 and D61 correlate with fast internal motions, τ_e , found in the β 3- α 3 loop. Elements of secondary structure: β 1-strand, K5-D10; α 1-helix, G14-K25; β 2-strand, Q29-A33; α 2-helix, G36-K45; β 3-strand, L50-D54; α 3-helix, I63-D73; β 4-strand, I76-T82; α 4-helix, Q91-G97; β 5-strand, T100-A103; α 5-helix, I108-L119. Phosphorylation occurs at D54 in an aspartyl pocket consisting of D54, D10 and D11.

temperature dependence. A tryptic digest-mass spectrometry analysis demonstrated the absence of deamidation or methionine oxidation products.

Of interest is the potential correlation between the dynamic properties of Spo0F and its function. Particularly striking is the overlap between the regions of the protein exhibiting significant R_{ex} values and those important for protein-protein interactions between Spo0F and other members of its signal transduction pathway (Fig. 3c). All the mutations affecting KinA, Spo0B and RapB interactions are isolated to the regions of the β 1- α 1 loop, α -helix 1, β 3- α 3 loop, β 4- α 4 loop and β 5- α 5 loop, regions that partially overlap to form a semicontiguous surface surrounding the site of phosphorylation (at D54). The residues affecting K_m for KinA are I15, R16, L18, K56, I57, M81, Y84, E86, L87 and I108 (ref. 8); Y13 and Y84 have been implicated in stabilizing Spo0F:RapB interactions through hydrophobic contacts⁹; and the regions important for Spo0B interactions, whether through K_m or k_{cat} effects, are isolated to the same residues⁸. The direct correlation to R_{ex} with most of these residues indicates a significant amount of conformational exchange in these backbone regions before any encounter with a binding partner.

Several functionally important residues close to the site of phosphorylation, including I15, L19, I57, M81 and F106, also have τ_e or R_{ex} . When mutated to alanine, each of these residues significantly enhances dephosphorylation by RapB and affects the stability of the phosphorylated state^{8,9}. These hydrophobic residues are significantly conserved throughout the response regulator family, are at least partially buried near D54 and are important in the structural integrity of this family¹⁷. The flexibility of these residues and their potential structural role implicate them as being important in the conformational change that occurs in Spo0F upon phosphorylation. In addition, the fluctuations are likely to be important in the protein's ability to interact with the other proteins in this signal transduction pathway, and provide the basis for proposals of mechanisms for these interactions.

The different conformers observed for the β 1- α 1 loop and α -helix 1 region indicate the structural detail of the mechanisms of Spo0F interactions with KinA, RapB and Spo0B. Residues D10-Y13 of the β 1- α 1 loop have some of the highest root mean square (r.m.s.) deviations and the largest R_{ex} values (Fig. 2c). The R_{ex} terms of 5-30 s⁻¹ correspond to regions of the backbone that deviate up to an angstrom in the ¹³C backbone (averaged over 20 structures). The NMR structures indicate that the side chains of Y13, R16 and I17

adopt multiple conformations and influence one another through a hydrophobic cluster formed at the β 1- α 1 loop and the N terminus of α -helix 1. Several side-chain positions for R16 are observed, ranging from a solvent-exposed guanidinium group, to one in which the guanidinium group extends into the aspartyl pocket (the site of phosphorylation) itself. Exchange between these positions does not seem to be hindered sterically from inspection of space-filling models of the structures. The phenolic ring of Y13 is packed against hydrophobic residues of the N-terminal region of α -helix 1, with the plane of the ring perpendicular to the methyl groups of I17, which further stabilizes interactions with the R16 side chain. RapB may catalyse dephosphorylation through constraining the motion of the β 1- α 1 loop, thereby allowing the guanidinium group of R16 to adopt a conformation in the aspartyl pocket that stabilizes the transition state for acyl-phosphate hydrolysis¹⁹. KinA and Spo0B use the residues of this region primarily to stabilize protein-protein interactions (mutations give K_m effects⁸); the conformational deformability observed for this region may be important in accommodating the binding of these different proteins at this surface. It is also plausible that KinA and/or Spo0B use the deformability of this region to influence a conformational change at the aspartyl pocket, thereby enhancing phosphoryl transfer.

The β 3- α 3 loop (M55-G62) immediately follows the site of phosphorylation (Fig. 3a) and here two motions are implied. First, the backbone (ϕ , ψ) angles suggest flexibility between G59 and G62, providing a twisting motion for the amides relative to one another. This correlates with the fast-timescale internal (τ_e) motions of M60 and D61. The large τ_e value observed for I57 may arise through side-chain hydrophobic interactions with M60, an interaction that is important in stabilizing the γ -turn-like structure of residues P58 to M60. The second motion is a 'flapping' of the entire loop from M55 to D61. This concerted motion is coincidental with the slower-timescale R_{ex} terms measured for amides of M55, K56 and D61 at the extremities. Mutation of these residues affects interactions with all three of the Spo0F binding partners and both K_m and k_{cat} for KinA interactions. The conformation of this loop must be important for recognition, binding stabilization and/or propagation of the conformational changes upon phosphorylation.

Finally, previous studies have shown that β -strand 5 of Spo0F has multiple conformers in slow exchange at pH 6.85 and that the β 4- α 4 loop, α -helix 4, β -strand 5 and K104 are influenced by this

conformational exchange¹⁰. Specifically, the imidazole ring of H101 adopts multiple conformations, one buried and hydrogen-bonded to the T82 amide and the other in a more solvent-exposed conformation. The large R_{ex} terms observed for residues T82, G85, K94, T100 and F102 are consistent with the timescale of slow exchange of the histidine ring in and out of the protein. The motion of H101 influences the conformation of the $\beta 4$ – $\alpha 4$ loop, affecting KinA and RapB interactions.

The paradigm of response regulator activation would require phosphorylation-induced conformational change to propagate out from the site of phosphorylation to the regions responsible for protein–protein interactions¹⁸. Our results provide evidence for a communication network that accomplishes this task. This model proposes that R16, M81, T82 and K104, which are close to the site of phosphorylation, functionally significant and exhibit a propensity for conformational change, are initially perturbed directly by phosphorylation. The perturbations are then transmitted to the general backbone regions where they reside, namely α -helix 1 (R16), the $\beta 4$ – $\alpha 4$ loop (M81, T82) and the $\beta 5$ – $\alpha 5$ loop (K104)¹⁹. In addition, the role of T82 is important in its interaction with H101 on β -strand 5 and the conformational exchange of the imidazole ring between the buried and exposed position¹⁰. Extending this model, the conserved, buried residues mentioned above (I15, L19, I57 and F106) may also be involved in transmitting structural perturbations to α -helix 1, the $\beta 3$ – $\alpha 3$ loop and the $\beta 5$ – $\alpha 5$ loop. The cluster of functionally important hydrophobic residues, I15, L19, M81 and F106, may also serve to alter the surface at the interface between α -helix 1 and α -helix 5. These loops comprise a surface that surrounds the phosphorylation site and movements by the residues in the loops or those buried below can alter the surface presented to other proteins. Such changes are likely to be important not only in recognition of the respective target but also in the dissociation from the kinase protein after phosphorylation.

That Spo0F should have a high degree of flexibility in the apo state may seem counterintuitive to our understanding of thermodynamics in protein–protein interactions. If complex formation between Spo0F and one of its binding partners favours a single Spo0F conformer, one might expect a large entropic cost relative to the free Spo0F form. In this study we have not examined the dynamic characteristics of Spo0F complexes. However, the surface identified as important for protein–protein interactions is highly hydrophobic. Burial of this surface upon complexation may serve to offset entropic energy loss. Additionally, from a functional perspective, an advantage may be gained by some entropic cost serving to prevent any given protein–protein interaction from becoming too energetically stable, allowing Spo0F to interact rapidly with its various partners. Phosphorylation may be a means of providing the energy for slight conformational changes required for protein dissociation. Previous observations of relative disorder to more ordered conformations in protein–protein, protein–peptide and protein–DNA complexes have led to similar suggestions that flexibility provides for diversity in binding partners^{20–22}. An increase in dynamics in the PLCC SH2 domain upon binding the phosphotyrosine peptide may also provide a means of modulating binding affinities²³.

Equilibrium fluctuations and functionally important fluctuations may be related²⁴. This presumes that the initial and final states are close in structure; the difference between them is a shift in the population of conformational fluctuations available. For example, a protein may sample a set of conformations in its ‘initial’ state and the activation of a protein may merely alter the structure to prefer one of the sub-populations observed in that state. Thus, the overall initial and final structural conformation between apo-Spo0F and phosphorylated Spo0F or between apo-Spo0F and Spo0F:KinA, Spo0F:Rap and Spo0F:Spo0B complexes, may be similar. Phosphorylation would then serve to shift the population of conformational substates. This view is supported by the apparent paradox

of recent crystal structures of response regulator complexes and structures of response regulators with ‘activating mutations’ that show few structural changes relative to the apo-forms^{25,26}. These structures contradict global changes in chemical shift detected by NMR that suggest that much of the protein undergoes at least minor conformational change (ref. 27 and V.A.F. and J.C., unpublished data). If the apo and ‘activated’ structures share functionally important fluctuations, then the crystallization process may indeed select out one population, not necessarily the ‘active’ conformers. Many response regulators have a low level of activity before phosphorylation, and the idea that the functionally important conformations are sampled in the apo-state and that phosphorylation serves to shift the population to the fully active conformation may assist in defining mechanistic models more precisely. □

Methods

Sample preparation. Uniformly ¹⁵N-labelled Spo0F was prepared by over-expression of the pET0F (ref. 28) containing *E. coli* strain BL21DE3 in M9 minimal media with ¹⁵N-NH₄Cl (Isotec). Spo0F was purified according to previously published methods²⁸ with one alteration. After elution from the DEAE-TrisAcryl column, combined fractions were brought to 35% (w/w) (NH₄)₂SO₄ and loaded onto a phenyl–Sephacryl column equilibrated in 25 mM Tris, pH 7.5, 50 mM KCl, 1 mM MgCl₂ and 35% (NH₄)₂SO₄. After washing, the protein was eluted with a 35–0% (NH₄)₂SO₄ gradient and a flow rate of less than 1 ml min^{−1}. Fractions were combined, dialysed against the final NMR buffer, concentrated to a 5 ml volume and loaded onto an S-100 Sephacryl gel filtration column (Pharmacia), equilibrated in NMR buffer. These substitutions decrease sample loss at the hydroxylapatite column step and enhance sample purity. The final sample conditions were 1.4 mM ¹⁵N-Spo0F, 10 mM potassium phosphate, 50 mM KCl, 0.02% Na₂S₂O₃, 10% D₂O, pH 6.80 ± 0.05. Mass spectroscopy of the protein demonstrated better than 99% purity and a relative molecular mass (M_r) of 14,390. Dynamic laser scattering spectroscopy (DLS; DynaPro) analysis of sample at 1.4 mM gave monophasic fit of the scattering and a molecular size consistent with a protein of M_r 14K.

Data collection. ¹⁵N relaxation parameters, R_1 and R_2 relaxation rate constants and steady-state ¹H–¹⁵N heteronuclear NOEs were obtained using experiments described²⁹ and modified for PEP-Z sensitivity enhancement and relaxation difference corrections³⁰ on a Bruker DRX 600 MHz spectrometer equipped with a triple-resonance, triple-axis gradient probe, at 300 K. R_1 experiments were collected with delay times of 18, 126, 252, 378, 576 and 900 ms, 1.26 and 2.61 s with duplicate spectra at time points 18, 252, 576 and 900 ms and a relaxation delay of 1.3 s (over two times the average T1 value). The R_2 measurements were collected at 9, 16, 32, 48, 72, 104, 160, 368 and 528 ms with duplicate spectra of time points 8, 72 and 160 ms. Duplicate NOE experiments were collected with a recovery delay of 3.0 s in both the on and off resonance experiments. ¹H–¹⁵N-HSQC were collected before and immediately after the collection of all dynamics data.

Data processing and analysis. Data was processed using Felix950 (Molecular Simulations) as described previously¹⁰. R_1 and R_2 rate constants and steady-state NOEs were determined from resonance peak heights as described¹⁴ using computer programs provided by M. Akke and A. G. Palmer. Uncertainty in NOE values was determined as described². Model-free parameters were fit using the FORTRAN program Modelfree, version 3.1 (provided by A. G. Palmer) according to the protocols described¹³. The τ_m optimization was tested by varying initial estimates between 9.5 and 5.0 ns and were found to converge. Analysis of the anisotropic diffusional tensors was done using the protocols described^{15,16} and the programs provided by L. Lee and A. G. Palmer.

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Correspondence and requests for materials should be addressed to J.C. (e-mail: johnhnc@wadsworth.org); address from 1 August 1999: Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, USA. Relaxation data are available as Supplementary Information and values for R_1 , R_2 , NOE, S^2 , τ_{ex} , R_{ex} SSE and models are deposited at the Indiana Dynamics Database (<http://pooh.chem.indiana.edu/IDD>).